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THE NUCLEAR MATRIX AFFECTS SATB1-MEDIATED MMTV SUPPRESSION

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by

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DISSERTATION

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Dedication

To my parents and my wife

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The graduate school years were not only a great journey in training as an independent scientist but also a time to learn to feel God who is always with me and talks to me.

THE NUCLEAR MATRIX AFFECTS SATB1-MEDIATED MMTV SUPPRESSION

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Mouse mammary tumor virus (MMTV) is a betaretrovirus that causes mammary adenocarcinomas in susceptible mouse strains. Milk-borne MMTV initially causes a low level infection of gut-associated lymphoid cells, which then deliver the virus to mammary epithelial cells, a target for high levels of MMTV replication and insertional mutagenesis. Although wild-type MMTV is not lymphomagenic, variants that cause thymic lymphomas have alterations in the long terminal repeat (LTR), including a deletion of negative regulatory elements (NREs). This deleted region contains binding sites for at least two cellular proteins, CCAAT displacement protein (CDP) and special AT-rich binding protein 1 (SATB1). SATB1 is expressed primarily in the thymus and has been proposed to suppress MMTV expression and integration in lymphoid tissues.

Because of previous difficulties with SATB1 overexpression in culture, a transcriptional assay system for SATB1 function was developed. Rat fibroblasts or mouse mammary cells, which express little SATB1, were stably transfected with

MMTV-luciferase reporter genes. Transduction with retroviruses expressing wild-type SATB1 suppressed MMTV LTR expression. SATB1 transduction also suppressed expression of integrated MMTV proviruses in rat cells, consistent with the primarily repressor function of this transcription factor.

To delineate domains responsible for DNA binding and suppression of MMTV transcription in this system, SATB1 mutants were generated. SATB1 is known to bind to the nuclear matrix, which has been shown to play important roles in transcription. A nuclear matrix targeting sequence (NMTS) of SATB1 was identified as a 55-amino acid region between the dimerization domain and the major DNA-binding domain. Both the DNA-binding and dimerization domains were necessary for binding to the MMTV NRE using gel shift assays, but the NMTS was not. Deletion of the DNA-binding domain or the NMTS significantly alleviated repression of stably integrated MMTV LTR-reporter genes, confirming the importance of both DNA binding and nuclear matrix binding for SATB1-mediated MMTV suppression. Additionally, cellular factors interacting with this SATB1 NMTS were identified through screening of a human thymic cDNA library using the yeast two-hybrid system. Further studies using this reporter system for SATB1 function and identification of protein interactions with the nuclear matrix should provide unique insights into transcriptional regulation and tissue-specific transcription.

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1. INTRODUCTION

1.1 MOUSE MAMMARY TUMOR VIRUS (MMTV)

1.1.1 Classification

The mouse mammary tumor virus (MMTV) was first identified in the 1930s as a causative agent of a high incidence of breast cancer in certain inbred mouse strains. MMTV is the prototype species of the genus *Betaretrovirus* within the family *Retroviridae*. The morphology of the virion core originally was used for subclassification within the family *Retroviridae*, and MMTV was classified as a B-type virus characterized by a round, but eccentrically positioned, inner core. There are two types of MMTV: exogenous MMTVs (milk-borne strains) and endogenous MMTVs. Exogenous MMTV strains are often named after the inbred mouse strain from which they were derived, for example, C3H MMTV or MMTV (C3H). Endogenous MMTV proviruses are specified as *Mtv* followed by a number, for example, *Mtv*8 (57,159).

1.1.2 Virion structure and genome structure

Retroviruses contain two identical copies of a single-stranded RNA. The genome is encapsidated by NC protein as a helical ribonucleoprotein (RNP) and is surrounded by CA proteins to make an icosahedral capsid. The capsid is attached to the envelope by the MA protein. Viral envelope is derived from the cellular plasma membrane and is embedded with a transmembrane (TM) protein and a surface (SU) protein. Within the internal core, there are a few molecules of virally coded, RNA-

dependent DNA polymerase (reverse transcriptase) and cellular tRNAs that are used as primers for reverse transcriptase.

The ends of the viral genomic RNA contain short direct repeats (R), which flank the virally encoded genes, *gag*, *dut*, *pro*, *pol*, *env*, *rem*, and *sag*, as well as non-coding sequences comprising unique sequences at the 5' (U5) and 3' (U3) ends of the genome (57) (Fig.1). The primer-binding site (PBS) is located just downstream of U5 and is used for binding of the cellular tRNA that primes negative-strand DNA synthesis by reverse transcriptase. The PBS is followed by the *gag* gene, which encodes MA, p21, CA, and NC (5' to 3'), the nonglycosylated internal structural proteins. The *gag* gene is followed by coding sequences for the *dut* and *pro* genes that encode a dUTPase and protease, respectively. The *pol* gene encodes RNase H, reverse transcriptase (RT), and integrase (IN), whereas *env* encodes the envelope proteins, SU and TM. Interestingly, another gene, *rem*, has been discovered with the *env* coding sequences. The *rem* gene appears to encode an RNA export protein (M. Mann and J. Mertz, manuscript in preparation). MMTV is one of the few retroviruses that encode a gene within the U3 region (164). The *sag* gene encodes a protein that is critical for milk-borne MMTV transmission from the gut of newborn mice to susceptible cells in the mammary glands.

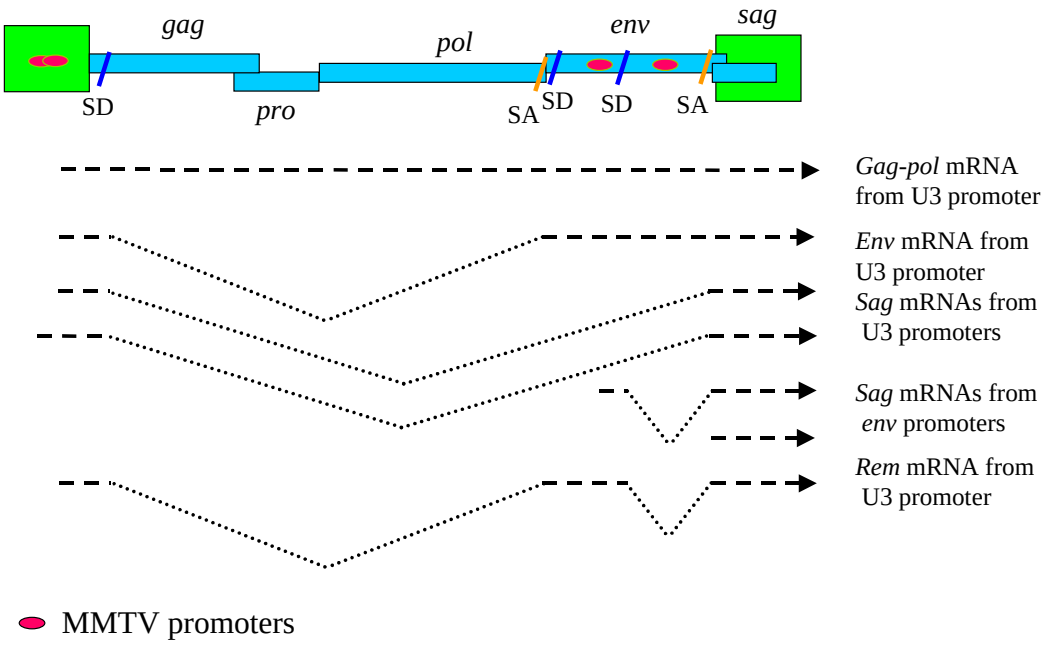


Fig.1. Diagram of MMTV RNAs from LTR.

Integrated MMTV proviruses transcribe RNAs from the 5' LTR promoter. The RNA is spliced to make *env*, *rem*, and *sag* mRNAs using the splice donor (SD) and splice acceptor (SA) sites. Functional *sag* mRNAs are made from the *env* promoter (146). Arrows show the direction of transcription. Thick dotted lines indicate the exons and V-shaped thin dotted lines indicate the regions removed by splicing. [This figure is adapted from (57,146).]

1.1.3 The MMTV life cycle

Exogenous MMTV is transmitted to newborn pups through mother's milk, whereas endogenous virus is transmitted by germline cells. The virus in milk must traffic through the gut of newborn pups to the mammary epithelial cells, the final target for MMTV infection (Fig. 2). MMTV uses lymphoid cells whose mobility allows distribution of the virus among various tissues. The virus present in the gut crosses M cells and infects B cells in the Peyer's patches of the gut. The B cells are activated and present virally encoded superantigen (Sag) associated with MHC class II protein at the cell surface. Interactions with specific Vβ chains of T-cell receptors activate the T cells to produce cytokines, which subsequently activate bystander cells to divide. These dividing cells are infected by MMTV and serve as a reservoir of infection for mammary gland cells during puberty (201).

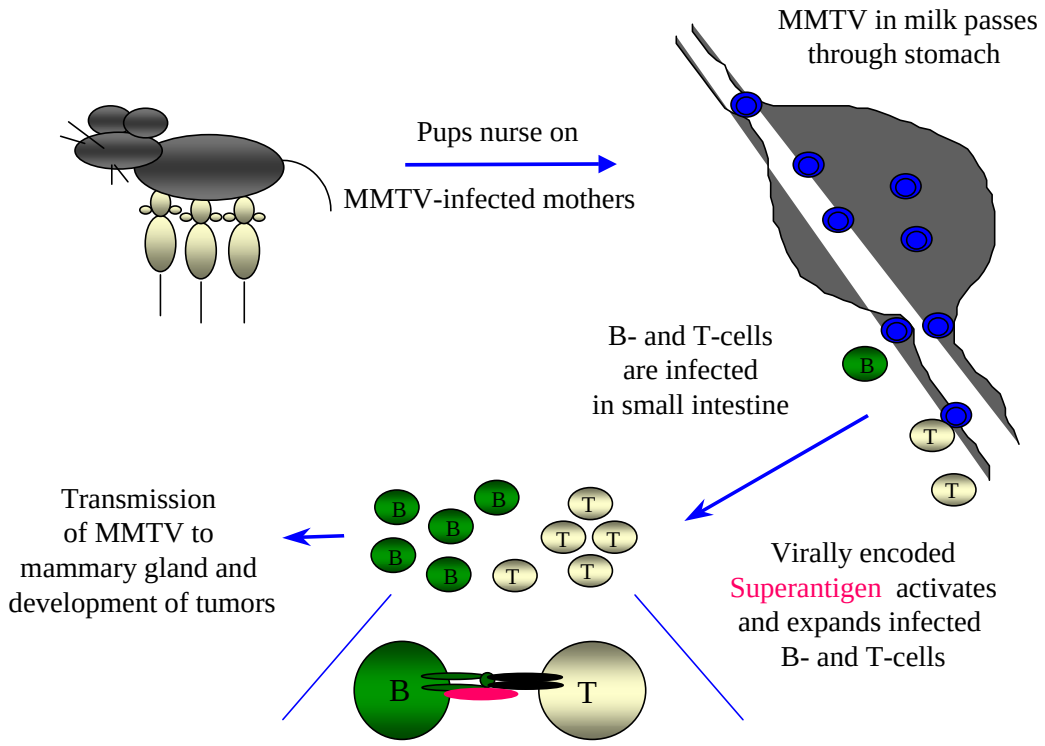


Fig. 2. Life cycle of milk-borne MMTV.

Newborn pups ingest milk containing MMTV from infected mothers. The virus passes through M cells in the gut prior to infection of B and T cells. The infected B cells present virally encoded superantigen (Sag) in conjunction with MHC class II protein at the cell surface. Sag presentation allows interaction with specific T-cell receptors to activate T cells that subsequently produce cytokines. These cytokines induce the division of bystander lymphoid cells that become infected by MMTV. Infected B and T cells are required to transfer virus to the mammary glands, the final target organ.

1.1.4 Virus attachment and entry into host cells

The binding of the envelope protein (Env) to cellular surface receptor(s) is the first step of viral infection. All mouse tissues bind to MMTV envelope protein, SU, although the binding affinity is different among tissues. Binding is highest in the mammary gland and spleen, intermediate in the ovary and salivary gland, and lowest in the liver and adrenal glands (11).

To identify the MMTV receptor, hybrids between Chinese hamster cells and mouse tumor cells were developed. These hybrid cell lines were infected with pseudotypes of vesicular stomatitis virus (VSV) containing MMTV envelope protein. After infection, analysis of the segregated mouse chromosomes in the mouse-hamster hybrids mapped this MMTV receptor (*Mtvr1*) to mouse chromosome 16 (85). A second cellular receptor for MMTV (*Mtvr2*) was identified as a novel transmembrane protein on the proximal end of chromosome 19 using a cDNA expression library (75). The discrepancy suggests that MMTV may have more than one receptor or a co-receptor for attachment and entry into susceptible cells. Most recently, the identity of *Mtvr1* was revealed to be the transferrin receptor (*Tfrc*), which is located on mouse chromosome 16 at 21.2 cM (178). The transferrin receptor is ubiquitously expressed on all rodent cells, and its ability to be recycled to the cell surface explains the lack of superinfection resistance observed with MMTV, but not many other retroviruses (61,178).

1.1.5 Virus replication

Retroviral replication is initiated by the reverse transcription of the single-stranded viral RNA genome into a double-stranded DNA copy. The DNA is

subsequently integrated into the host cell chromosomal DNA mediated by virally encoded integrase (211). RT synthesizes a minus-strand DNA from plus-stranded viral RNA using its RNA-dependent DNA polymerase activity. The RNA portion of the RNA-DNA hybrid is degraded through the RNase H activity. RT then synthesizes a plus-strand DNA using the minus-strand DNA template, its DNA-dependent DNA polymerase activity, and RNA primers derived from the degraded plus-stranded RNA. The resulting double-stranded DNA or provirus forms viral nucleoprotein complexes associated with IN, which is required for integration (57). The nucleoprotein complexes associate with cellular chromosomes when the nuclear membrane breaks down during cell division. IN removes a dinucleotide from the ends of proviral DNA to produce 3'-OH recessed LTRs. The two recessed ends are integrated into host chromosomal DNA by a concerted mechanism in which both viral DNA ends attach to sites on opposite strands of the target DNA. However, like other retroviruses, MMTV integration occurs at many sites in the cellular DNA (57).

The integrated provirus contains many regulatory cis-acting elements within the LTR, including binding sites for RNA polymerase II. Transcription by RNA pol II starts at the U3/R junction within the 5' LTR and terminates at the R/U5 junction within the 3' LTR. Transcripts are capped and polyadenylated by cellular enzymes in the nucleus. Some portions of the full-length transcripts are transported into the cytoplasm without splicing for translation of the Gag and Pol proteins or are packaged into new virus particles. Other transcripts are spliced to generate the singly spliced *env* and *sag* mRNAs or the doubly spliced *rem* mRNA.

1.1.6 Transcriptional regulation

1.1.6.1 Hormone-responsive element (HRE)

MMTV transcription is elevated 10- to 50-fold when infected cells are grown in the presence of glucocorticoids or other specific steroid hormones, such as progesterone and testosterone (174). Four binding sites for ligand-bound hormone receptors reside in the hormone-responsive elements (HREs) between –200 and –50 relative to the transcription initiation site in the MMTV LTR (91,114,161,181,181,206). Sequence analysis of these regions showed that the hexanucleotide 5'-TGTTCT-3' represents receptor binding sequences (181). Hormonal induction is achieved by the loading of transcription factors, including NF1, OCT1 (OTF1), and TFIID downstream of the HRE in the MMTV LTR (200), subsequent to the transient interaction of ligand-bound steroid receptors with their binding sites (7,82).

Ligand-dependent transcriptional activation by nuclear receptors is achieved by recruitment of complex co-regulators on the promoters of hormone-regulated genes (113). These receptors directly interact with general transcription factors (GTFs), such as TATA-binding protein (TBP) and TBP-associated factor ($TAF_{II}110$), a subunit of TFIID, to initiate transcription (182,183). However, stimulation of reporter gene transcription by the progesterone receptor is inhibited by co-expression of the estrogen receptor (138). The result indicates that there is competition between nuclear receptors and limited transcription factor(s). However, the transcriptional squelching between receptors cannot be explained solely by the direct interaction between nuclear receptors and GTFs. A search for nuclear receptor co-activators revealed two groups

of co-activators, known as p300/CREB-binding protein (CBP) and the mammalian Mediator complexes (TRAP, DRIP, ARC, etc.) (24,169).

Interactions between p300/CBP and the nuclear receptors can occur directly or indirectly through members of the steroid receptor coactivator (SRC) family, such as SRC-1, TIF2/GRIP1, and RAC3/ACTR/pCIP/AIB-1 (33,119). Nuclear receptor interactions with p300/CBP are necessary for their transactivation function (1,33,100). p300 and CBP are large nuclear proteins, which have been demonstrated to interact with many transcription factors, including CREB, AP1, NF-κB, and SATB1, as well as general transcription factors, such as TFIIB and TBP (66,76,96,163). Therefore, p300/CBP proteins link components of the basal transcription machinery and specific DNA-binding transcription factors. Moreover, these co-activators have intrinsic histone acetyltransferase (HAT) activity (156). This observation implies that p300/CBP may contribute directly to transcriptional regulation via targeted acetylation of chromatin as well as by serving as a simple adapter between transcription factors and p300/CBP-associated factor (P/CAF) or GTFs (217).

Mediator first was identified in yeast as a complex that was associated with RNA Pol II and required for transcriptional activation *in vitro* (147). The metazoan versions of yeast Mediator are central and necessary components for transcriptional activation by many transcription factors (169). Thyroid hormone receptor-associated protein (TRAP) and Vitamin-D-receptor interacting protein (DRIP) complexes were identified as human Mediators that interact with nuclear receptors (93,170). The mammalian Mediator complexes are required for activator-responsive transcription and

usually consist of heteromeric complexes containing from 7 to 25 distinct polypeptides (24,169).

1.1.6.2 Negative regulatory elements (NREs)

Nucleotide sequences of proviruses acquired in MMTV-induced mouse T-cell lymphomas showed 400 to 500 bp deletions in the U3 region (located from ca. -655 to –165). Furthermore, an MMTV LTR-reporter gene carrying an NRE deletion exhibited higher transcriptional activity in mink lung cells than that of wild-type LTRs, suggesting that the deleted region contains NREs (58,89). Mice carrying MMTV LTR-reporter plasmids with NRE deletions showed expression in novel sites, such as the heart, brain, and skeletal muscle, where endogenous MMTVs and the full-length LTR are not expressed (177). Thus, negative elements in the MMTV LTR suppress viral transcription in many different tissues.

To further map the sequences responsible for tissue-specific transcriptional suppression, a series of deletion mutants were prepared in the LTR region between -634 to -158. Experimental results using these mutants verified that there are two negative elements: one in the region from -484 to -426 designated the promoter-distal NRE, and one in the region between -281 to -255 designated promoter-proximal NRE (25). Gel shift assays showed that two high-molecular-mass complexes bound to both NREs, and these complexes were termed upper or ubiquitous-binding protein (UBP) and NRE-binding protein (NBP). Protein purification experiments and gel shift/antibody ablation assays identified special AT-rich binding protein 1 (SATB1) as NBP and CCAAT displacement protein (CDP) as UBP (124). Mice carrying an MMTV LTR-

reporter gene with a SATB1-binding site mutation (a 7-nt substitution mutation from -272 to -265) showed an altered tissue-specific distribution of MMTV expression. A dramatic increase in transcription was observed from mutant LTRs in lymphoid tissues where MMTV expression is normally suppressed, relative to mammary tissue, which normally shows the highest MMTV expression (25,124).

The MMTV LTR has multiple CDP-binding sites, and overexpression of CDP decreased MMTV LTR-reporter gene expression in transient transfection assays. Mutations of CDP-binding sites in the LTR alleviated suppression of MMTV transcription in both stable and transient transfections, and wild-type CDP suppressed MMTV-reporter gene expression in the presence or absence of dexamethasone (DEX) (226,227). Furthermore, CDP and SATB1 are both homeodomain proteins that are members of the Cut repeat family (123). These proteins appear to interact with each other to regulate binding of each protein to the MMTV NRE. Overexpression of CDP in Jurkat T cells that express high levels of SATB1 was able to elevate expression of a co-transfected MMTV LTR-reporter plasmid (123). These results suggest that tissue-specific MMTV expression may be partially controlled by the levels of two different repressors, CDP and SATB1 (123).

1.1.6.3 Mammary gland enhancer element

Mice expressing an MMTV LTR-*myc* transgene developed breast tumors at an accelerated rate, suggesting that LTR directly influences mammary-specific transcription (193). MMTV LTR transgenes lacking sequences between -863 and -110 showed tissue-specific expression similar to that of the full-length MMTV LTR,

suggesting that the 5' end of the LTR is important to direct transcription in breast tissues (192). A fragment of the MMTV LTR from -1166 to -987 was fused to a chloramphenicol acetyltransferase (CAT)-reporter gene and used to generate transgenic mice. These transgenic mice showed elevated CAT activity in both mammary and salivary gland, suggesting that this LTR region acts as an enhancer of transcription in these tissues (142). In transfection experiments, the region of the MMTV LTR from -1075 to -978 proved to be 5-fold more active in a mammary cell line (34i) than in NIH3T3 fibroblasts (136). Furthermore, six *cis*-acting elements in this enhancer were identified, and multifactor complexes, such as mp5/AP-2 and NF-1/CTF, bound to these elements to control enhancer activity (117,136). Stat5 or related proteins bind near +520 (-675) on the MMTV LTR (195). Stat5a- or Stat5b-knockout mice showed reduced MMTV expression in mammary glands, but not in lymphoid tissues, demonstrating that this transcription factor family also is important for mammary-specific transcription (168).

1.1.6.4 Chromatin remodeling in the MMTV LTR

Eukaryotic DNA is compacted into chromatin affecting the accessibility of DNA-binding proteins (7). The basic unit of chromatin, the nucleosome, is formed by core histones with a $(H3\text{-}H4)_2$ tetramer flanked by two H2A/H2B dimers (127). A well-characterized nucleosomal structure has been observed on the integrated MMTV LTR (173), which has been studied using cell lines containing bovine papillomavirus (BPV) episomes carrying the MMTV LTR. Nuclei from cells carrying the MMTV LTR on BPV episomes were digested with micrococcal nuclease or with the chemical MPE-Fe(II) to

study nucleosome positioning. The two digestion methods showed the same cleavage pattern, which differed from that of the naked MMTV LTR. Cleavage sites were localized at +136, -60, -250, -444, -651, -826, and -1019 relative to the transcriptional initiation site with spacing of ca. 180 to 200 bp, consistent with nucleosomes (Fig. 3). Furthermore, in the presence of a synthetic glucocorticoid hormone, dexamethasone, the cleavage sites were not altered except for the region between -60 and -250 containing the HRE (173).

The synergistic binding of glucocorticoid receptor (GR) and transcription factors to the MMTV LTR has been well studied. Nuclear factor 1/CCAAT-transcription factor (NF1/CTF) and GR bind to one of the nucleosomes (Nuc-B) in the MMTV LTR (47). The activation of the MMTV promoter by GR is thought to be a bimodal process based on experiments with both stable and transient templates. NF1 was excluded from its binding site on stably transfected MMTV promoter without glucocorticoid treatment. However, the transcription factor constitutively binds to the transiently transfected MMTV reporter in the absence of hormones, suggesting that the naked template is more accessible to transcription factor binding. The bimodal model involves (i) GR-dependent structural transition of the chromatin, which leads to NF1/CTF loading, and (ii) GR-mediated recruitment of other transcription factors. In this model, NF1 acts as an amplifier of the activation process (7).

The MMTV LTR promoter isolated in low-salt nuclear extracts showed three distinct forms: the transcriptionally inactive promoter, the basally active promoter, and the fully activated promoter. The inactive promoter contained TBP and histone H1. In contrast, the basally active promoter was bound by RNA pol II, TBP, Oct1, and

acetylated H3 in the absence of H1. The hormone-activated promoter contains BRG1, the catalytic subunit of the SWI/SNF remodeling complex, as well as the factors bound to the basally active promoter. All three forms condense into higher-order chromatin structures *in vitro* in the presence of Mg^{2+}. This suggests that the MMTV LTR promoter retains the ability to form the secondary/tertiary structure regardless of the binding of H1 and transcription factor complexes (69).

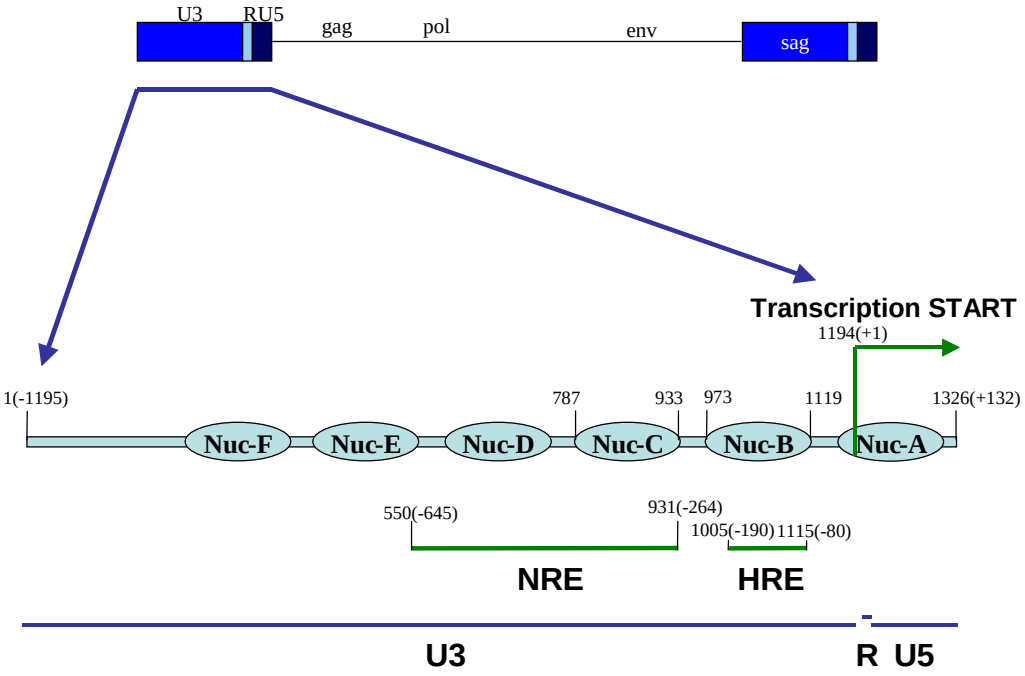


Fig. 3. The nucleosomal structure of the MMTV LTR.

The MMTV LTR is organized into a discrete nucleosomal structure. The top figure shows the proviral structure while the bottom figure shows the detailed nucleosome structure in the LTR. The LTR is shown as a box in the top diagram. Each nucleosome is shown as an oval and labeled Nuc-A to Nuc-F from the 3' to the 5' ends of the LTR. The numbers depict the position of each nucleotide relative to the first nucleotide of the LTR. The numbers in parentheses indicate the position of nucleotides relative to the transcription start site at the U3/R junction. Nucleosomes are positioned as follows: Nuc-A, +1159 to +1304; Nuc-B +973 to 1119; Nuc-C, +787 to +933; Nuc-D, +601 to +747; Nuc-E, +415 to +561; and Nuc-F, +229 to +375. The transcription start site, U5, R, U3 regions are also shown in the detailed nucleosome diagram. [Adapted from (6)]

1.1.7 Viral genetics

MMTV transmission occurs horizontally through exogenous (milk-borne) viruses and vertically through endogenous viruses. More than 30 germline-associated MMTV loci (*Mtv*) have been identified, and most mouse strains genetically transmit two to eight distinct *Mtv*s. However, the mammary tumor incidence of mice is primarily determined by transmission of virulent strains of milk-borne virus. The high-mammary cancer-incidence strain, C3H, has a 90% tumor incidence, while the BALB/c strain has a low-mammary-tumor incidence of 1% or less, yet both strains carry three or more endogenous *Mtv*s. The mammary tumor frequency of female C3H mice can be reduced by nursing newborn pups on BALB/c foster mothers. The resulting strain, C3HfBALB/c, has a 40% mammary tumor incidence. Conversely, the reciprocal strain, BALB/cfC3H, has a high incidence of breast cancer. Further experiments have established that C3H mice have a virulent milk-borne virus called C3H MMTV or MMTV(C3H) as well as a replication-competent endogenous provirus, *Mtv1* (57).

Both endogenous and exogenous MMTVs encode a superantigen (Sag) in their LTRs (109). Sag is important for trafficking of exogenous virus from the gut of susceptible pups to the mammary epithelial cells (74). The LTR sequences of different MMTVs are relatively conserved, yet the region encoding the C-terminal region of Sag is hypervariable, allowing various MMTV Sag proteins to interact with different subsets of T cells (212,219). Endogenous MMTVs that express Sag in the developing thymus eliminate Sag-reactive T cells, which are required for infection by exogenous viruses with same Sag specificity (84).

Host factors in different strains also influence susceptibility to disease induced by MMTV infection. C3H/HeJ mice have a greatly increased latency for mammary tumor induction after infection with milk-borne MMTV (C3H) relative to other substrains of C3H mice. These mice are highly susceptible to bacterial lipopolysaccharide (LPS) because they are unable to mount innate immune responses to this molecule. Recently, the *lps* gene was cloned and shown to encode toll-like receptor 4 (TLR4) (166). C3H/HeJ mice carry an attenuated milk-borne MMTV that appears to be the result of genetic selection for an altered immune response to the virus (158). C57BL mice also are resistant to MMTV-induced disease. These mice lack MHC class II I-E chains (143), which are necessary for efficient Sag presentation and function during MMTV trafficking to the mammary gland. I/LnJ strain mice represent another inbred mouse strain with genetic resistance to MMTV infection. These animals fail to develop mammary gland infections due to high circulating levels of neutralizing antibody to MMTV (73).

1.1.8 Pathogenicity

MMTV develop hyperplastic alveolar nodules (HANs) or plaques that often result in mammary carcinomas (133,216). There is a long latency between milk-borne viral infection from the gut and the development of mammary tumors, suggesting that MMTV does not contain an oncogene. Several investigators have shown that tumors arise due to multiple insertions near cellular oncogenes. *Int1* was the first common integration site to be identified in MMTV-induced tumors (153). Gene structure and nucleotide sequence indicated that *Int1* encodes a highly conserved growth factor that

is involved in wing development in *Drosophila* (9,204) and, therefore, the hybrid name *Wnt1* was derived. Many other common integration sites have now been identifid for MMTV-induced mammary tumors, including genes encoding many growth factors of the Wnt or Fgf families: *Fgf3* (*Int2*), *Notch4* (*Int3*), *Wnt3* (*Int4*), *Cyp19a1* (*Cyp19/Int5/IntH/aromatase*), *Fgf4* (*Hstf1/Fgfk*), *Fgf8*, *Eif3s6 (Int6),* and *Wnt10b* (154).

MMTV also induces T-cell lymphomas. MMTV proviruses from T-cell lymphomas invariably contain 350 to 500 bp deletions in the negative regulatory elements (137,213). Some MMTV proviruses isolated from lymphomas also contain duplicated sequences that have been shown to contain T-cell enhancers (137). Chimeric MMTVs in which the LTR from a mammotropic MMTV clone was replaced by the rearranged LTR from a lymphomagenic MMTV caused lymphomas in BALB/c mice, whereas the mammotropic clone induced mammary tumors only (213,214). Additional experiments have shown that the ability to induce lymphomas maps to the NRE and duplicated sequences in the U3 region. These results indicate that the tissue specificity of MMTV-induced disease is determined by the U3 region of the LTR.

1.2 THE NUCLEAR MATRIX

1.2.1 Identification of the nuclear matrix

Eukaryotic cell nuclei are mainly composed of DNA and histone complexes. However, the elution of these elements by high-salt extraction does not disrupt nuclear structure. The residual structure that retains the shape and some morphological features of the nuclei after sequential extraction of nuclei with detergents, nucleases, and high salt was originally defined as the nuclear matrix (16). A similar nuclear

remnant extracted with a mild ionic detergent, lithium 3, 5-diiodosalicylate (LIS) was labeled the nuclear scaffold (141); however, nuclear cage, nuclear ghost and nucleoid core are different names for the similar structures (95). Nuclear matrices are composed of a nuclear lamina with residual nuclear pore complexes, residual nucleoli, and an internal network of filaments connecting the residual nucleoli with the lamina (18) (Fig. 4). Therefore, the nuclear matrix consists of a peripheral lamina-pore complex and an intricate internal fibrogranular structure.

1.2.2 Nuclear matrix proteins

Initial analysis of the nuclear matrix showed that lamins are the major components of the lamina (15). Later, DNA topoisomerase II and nuclear mitotic apparatus protein (NuMA) were identified as constituents of the nuclear matrix (19,128). Subsequent studies by *in vivo* labeling with [^{35}S] L-methionine and two-dimensional electrophoresis showed that the nuclear matrix contains more than 200 different proteins. However, the composition of the nuclear matrix varied depending on the cell type. Twelve major proteins from rat liver nuclear matrix were separated and purified by peptide mapping and identified by antibody cross-reactivity. Five of these proteins were shown to be lamins A, B, and C, the nucleolar protein B-23, and core heterogeneous nuclear ribonucleoproteins (hnRNPs). The remaining proteins were unknown and were defined as the nuclear matrin (150). Recently, a new database that contains 398 nuclear matrix proteins, was construced through analyzing over 3000 scientific articles in PubMed (139).

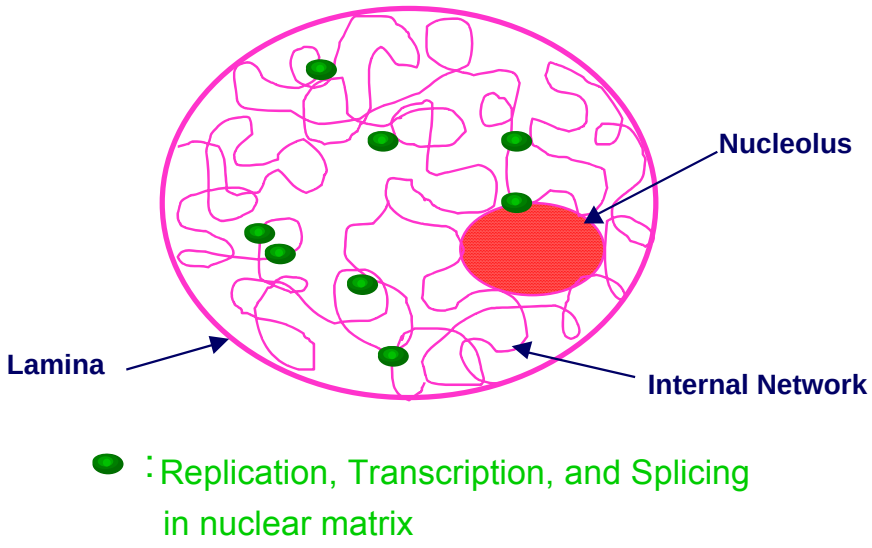


Fig. 4. Diagram of the nuclear matrix.
The nuclear matrix is defined as the residual structure following extensive extraction of chromatin and most soluble proteins. The diagram represents the nuclear matrix structure. The nuclear lamina define the outermost positions of the matrix. Inside the lamina, internal filament networks and the nucleolus are interconnected by lamina. Ovals indicate the sites that are important for nuclear reactions such as transcription, replication, and splicing.

Interestingly, it was shown that malignant cells express a different composition of nuclear matrix proteins compared to normal cells, and some researchers have found such proteins to be useful as cancer cell markers (27,70,126). Recently, mass spectrometry has been used to identify new matrix proteins (155).

Networks among the matrix or other regulatory proteins are formed to maintain the integrity of the nuclear matrix and to regulate important nuclear reactions. Nuclear

lamins, members of the intermediate filament protein family, are an example of this. They are integral components of the nuclear lamina, providing the structural framework for the nuclear matrix, as well as functioning as a regulator of transcription and splicing (90,116).

Keratin 19, one of the nuclear matrix intermediate filaments, is expressed in a tissue and differentiation-specific manner. This protein binds to human RNA polymerase II subunit 11, implying a relationship between nuclear matrix and transcription (28). One nuclear receptor, GR, which binds to the nuclear matrix, is important for the hormonal regulation of many cellular and viral promoters, including the MMTV LTR. The nuclear matrix targeting sequence (NMTS) of GR was determined to be a DNA-binding domain linked to the tau2 transactivation domain. One of the nuclear matrix proteins, hnRNP U, was shown to be responsible for GR binding to the nuclear matrix (199).

1.2.3 Nuclear matrix and loop structure

Chromatin fibers of chromosomes are organized by attachment to the nuclear matrix to form 30- to 100- kbp loop structures (2,103,118,160). The attachment sites are called the matrix/scaffold attachment regions (MAR/SARs), which are long stretches (200-800 bp) of AT-rich DNA containing consensus recognition sequences for topoisomerase II binding (21,22). MARs have been shown to be favored sites for histone H1 binding *in vitro* and may play a role in H1-dependent chromatin repression (102,188). It has been suggested that MARs also act as facilitators of replication by modulating chromatin architecture. The amplified dihydrofolate reductase domain

(amplicon) in CHOC 400 cells (140) contains MAR sequences (54). The replication origin regions in the amplicon undergo dramatic alterations in micrococcal nuclease hypersensitivity in a cell-cycle dependent manner when copies of the amplicon are affixed to the nuclear matrix (162).

MARs act as important transcriptional regulators by regulating chromatin structure (79). For example, MARs that flank the immunoglobulin heavy-chain enhancer are necessary to activate the distal variable-region promoter in transgenic mice (44,98). Mechanistically, it has been suggested that MARs regulate chromatin structures because most of the HAT and HDAC activities are associated with the nuclear matrix (49). Furthermore, MARs enhanced the acetylation of histones at the immunoglobulin heavy-chain enhancer distal nucleosomes, suggesting that MARs function to mediate long-range chromatin accessibility and transcriptional activation (63). Moreover, transcription can be regulated by MAR-binding proteins. This regulation is exemplified by the role of B cell regulator of IgH transcription (Bright) on gene expression from the immunoglobulin heavy chain intronic enhancer (101) as well as the ability of SATB1 to influence expression of a reporter gene flanked by MAR sequences (111).

1.2.4 The role of the nuclear matrix in transcription

A large body of evidence from different experimental systems suggests that the nuclear matrix plays an important role in transcription, perhaps by serving as a concentration site for transcription factors. Solubilized nuclear matrix proteins specifically bind to sequences within MARs, suggesting that sequence-specific DNA-

binding proteins localize in the nuclear matrix (60). Nuclear matrix proteins from HeLa S3 cells and rat ROS 17/2.8 cells have been shown to contain proteins that bind to well-characterized consensus sequences of Sp1, ATF, CCAAT, C/EBP, Oct1 and AP-1 (101). A developmentally important regulator, Pit-1 activates prolactin, growth hormone, and its own gene, *pit1*, and is localized in the nuclear matrix. However, some point mutations leading to dwarfism change its nuclear localization, suggesting that transcription factor activity is associated with nuclear architectures (129).

Fractionation studies show that transcriptionally active DNA is tightly associated with the nuclear matrix, whereas inactive loci are not. The ovalbumin gene in the chicken oviduct is associated with the nuclear matrix when the gene is stimulated by steroid hormones. However, when the hormone is withdrawn, the gene is detached from the nuclear matrix (42). The integrated polyoma and avian sarcoma virus (ASV) genomes in transformed cells also are associated with the nuclear matrix, but two subclones that lost the transformed phenotype and contained no detectable viral transcripts showed little association with the nuclear matrix (46).

Transcription complexes, including nascent transcripts with active RNA polymerases, are known to be associated with nuclear matrix that has been isolated under isotonic conditions (94,105). The finding that actively transcribed genes are associated with the nuclear matrix suggests that the nuclear matrix is involved in the global organization of chromatin.

Chromatin is organized into loops of heterogeneous size and the bases of these loops bind to the nuclear matrix through MARs/SARs. When the 5'-MAR of the chicken lysozyme-gene was linked to a reporter gene, the reporter expression was

significantly elevated regardless of the integration sites in its stable transfected cells (194). When the human interferon-beta (huIFN-β) gene was flanked by SARs, transcription increased 20- to 50-fold compared with the gene without SARs. Furthermore, transcriptional activation by SARs was orientation- and somewhat distance-independent (108).

Steroid receptors, such as the androgen receptor and the glucocorticoid receptor, bind to the nuclear matrix (205). Highly purified GR has been shown to bind to the nuclear matrix *in vitro* (107), but binding is dependent on the ATP concentration in the nucleus. GR binding to the nuclear matrix is dramatically increased by the depletion of ATP. However, GR is released into the soluble nuclear compartment with restoration of normal ATP levels (198).

1.2.5 Nuclear matrix role in replication and splicing

Several lines of experimental evidence suggest that the nuclear matrix plays important roles in DNA replication. Labeling of newly synthesized DNA with [^{3}H] thymidine showed that the nuclear matrix is associated with 90% of the newly synthesized DNA found in rat liver (17). Active DNA replication enzymes, including DNA polymerase α (190), DNA primase, 3' to 5' exonuclease, RNase H, and DNA methylase, are associated with nuclear matrix isolated from regenerating rat liver (203).

Replication origins are not permanently associated with the nuclear matrix (55). However, the association is controlled in a cell cycle-dependent manner. That is, replication origins associate with the nuclear matrix in late G1 phase and dissociate after initiation of replication in S phase (56). Immunolabeled DNA sites show that each

cell has approximately 150 focal sites of synthesis. The number of foci remains constant, even in cells from which 90% of the chromatin has been removed, suggesting that DNA replication is occurring on the nuclear matrix (87).

The splicing machinery is also associated with the nuclear matrix. Nuclear-mitotic apparatus protein (NuMA) colocalizes with splicing factors and is associated with small nuclear ribonucleoproteins (snRNPs), consistent with the relationship between RNA processing and the nuclear matrix (222). Furthermore, an intron-containing segment of the rabbit globin gene was transfected into HeLa cells to determine whether splicing occurs on the nuclear matrix. The reporter pre-mRNA, its splicing intermediates, and the resulting products associated with the nuclear matrix (221). When the nuclear matrix was incubated with *in vitro* HeLa splicing extract, the amount of matrix-associated precursor decreased, and the corresponding free intron lariat increased (221).

The nuclear matrix is also involved in the transportation of processed RNAs between subnuclear compartments. In fractionation experiments, spliced introns and pre-mRNAs of placenta and embryonic expression protein (*Pem*), a homeobox transcription factor, are present in the nuclear matrix fractions as well as in the high salt-soluble and chromatin-associated fractions (43). This result suggests that transcription and RNA processing occur in the same nuclear compartments, including the nuclear matrix. (43). Moreover, E1B 55kD protein encoded by adenovirus type 5 binds to the nuclear matrix in an E4 Orf6 protein-regulated manner and facilitates subnuclear RNA transport (120,121).

1.3 SPECIAL AT-RICH BINDING PROTEIN 1

1.3.1 Identification of SATB1

Repeats of sequences derived from the MAR located 3' of the mouse immunoglobulin μ heavy chain gene enhancer were used to screen a human testis cDNA expression library. From this screen, a new MAR-binding protein of 763 amino acids was cloned and identified as special AT-rich sequence binding protein 1 (SATB1) (53). SATB1 is expressed in a tissue-specific manner, primarily in thymus. Furthermore, this factor binds to AT-rich MAR sequences, but not to AT-rich non-MAR sequences (53).

Mouse SATB1 cDNA was cloned to determine the conservation of the SATB1 gene among different species. The murine protein has 764 amino acid residues with 98% homology to the human SATB1 sequence (149). A novel motif, a symmetric structure with a loop containing two short stretches of hydrophilic amino acids, was defined in the 150 amino acid MAR-binding domain (149).

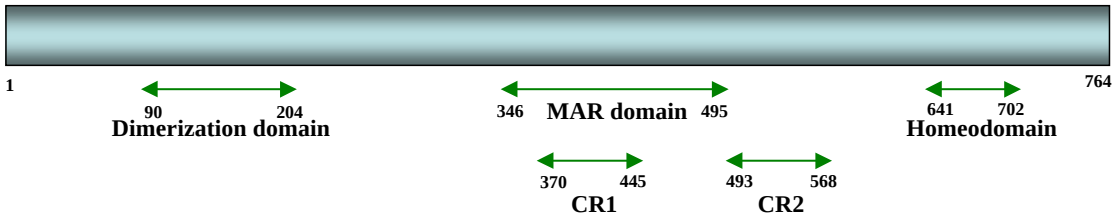


Fig. 5. Schematic diagram of SATB1

Positions of the dimerization domain, the MAR-binding domain, the Cut-like repeats (CR1 and CR2), and the homeodomain are indicated. [This figure is adapted from (52,68).]

1.3.2 Functional domains

To understand the mechanism of SATB1 action, functional domains of the protein have been identified. SATB1 contains a dimerization domain at the N-terminus followed by two Cut-like repeats, a MAR binding domain near the middle of the protein, and a homeodomain at the C-terminus (Fig. 5). It was originally suggested that SATB1 binds to DNA as a monomer, but subsequent experiments have shown that SATB1 functions as a homodimer. Deletion of the dimerization domain abolishes DNA binding, although the truncated protein has an intact MAR-binding domain and a homeodomain (68). The homeodomain per se does not bind DNA, but acts with the MAR-binding domain to participate in specificity of association with the core-unwinding element within the base-unpairing region (52).

SATB1 has been reported to recognize a specific sequence context in which one strand consists exclusively of mixed A, T and C nucleotides (ATC sequences) (53). AT-rich sequences in MARs have a strong tendency to unwind by extensive base unpairing (110). However, one SATB1-binding site in the MMTV LTR has G residues (124). SATB1 binding is highly specific and does not bind to similar AT-rich sequences lacking these unwinding capabilities. Moreover, this factor recognizes the minor groove of DNA with little contact with the bases (53).

To delineate the MAR-binding motif of SATB1, a random nanomeric peptide library constructed in a bacteriophage was incubated with the concatemerized 25-bp SATB1 recognition sequence from the IgH MAR. A peptide sequence (RQNWGLEGC) was selected as the MAR-binding motif. This peptide has 50% identity with a SATB1

sequence (amino acids 355-363), and replacement of the wild-type sequence with the new MAR binding motif in SATB1 gave full MAR-binding activity (207).

1.3.3 Interacting proteins

To further elucidate SATB1 function, a number of interacting proteins have been identified. A novel variant of RNA polymerase II, subunit 11 (RPB11) was obtained from a Jurkat T-cell cDNA library through yeast two-hybrid screening with SATB1. SATB1 interacts with RPB11 through the dimerization domain (PDZ domain) (59). SATB1 binds to CCAAT displacement protein (CDP), which is both a repressor and an activator of many cellular genes, depending on the promoters. Both SATB1 and CDP act as repressors on the MMTV promoter, but interactions between SATB1 and CDP abolishes DNA binding, thus relieving the transcriptional suppression (123). The X-linked lymphocyte-regulated (Xlr) protein, which is expressed in late stage B-lymphoid cell lines, also colocalizes with SATB1 in pre-T lymphocytes (62). These results confirm a role for SATB1 in transcription regulation, particularly in the lymphoid cell lineage.

SATB1 also interacts with several proteins involved in chromatin remodeling. Co-immunoprecipitation with ISWI, Mi-2, MTA-2 and HDAC1 suggests that SATB1 assembles protein complexes containing factors found in CHRAC/ACF and NURD complexes. The factors, hSNF2H and hACF1, components of the CHRAC/ACF remodeling complex, and HDAC1 directly interact with SATB1. Multimerized BUR elements from a MAR 3' of the immunoglobulin heavy chain enhancer were used in an affinity column to purify SATB1 and SATB1 binding proteins in thymus extract. SATB1

was copurified with the NURD (nucleosome remodeling and histone deacetylase) complex that has been implicated in transcriptional repression in several genes. The NURD complex contains ATP-dependent remodeling enzyme Mi-2, histone deacetylase 1 and 2, histone deacetylase associated co-repressor mSin3A, and MTA-2. In addition to the NURD complex, SATB1 binds to nucleosome-dependent ISWI-ATPase and ACF1, both subunits of the CHRAC/ACF complexes. Since SATB1 is bound to the bases of chromatin loops, these interactions may indicate that SATB1 targets chromatin remodeling complexes to specific nuclear locations to regulate gene expression (218).

1.3.4 SATB1-knockout mice

SATB1-null mice are smaller and thinner than their littermates and usually die within 4 weeks of birth. These animals exhibit neurological defects, such as incomplete eye opening and the clasping reflex. In addition, they have small thymi, spleens and lymph nodes with histologic abnormalities, and the number of thymocytes is reduced 10- to 100- fold by 3 weeks. These characteristics of SATB1-null mice are consistent with the fact that SATB1 mRNA is expressed primarily in thymus as well as fetal brain. Of the nearly 600 genes surveyed in SATB1-null thymocytes, approximately 2% showed differences in expression. Nine of ten genes tested by RT-PCR had increased expression, including c-*myc*, consistent with a primary role for SATB1 in transcriptional repression. The dysregulation of multiple genes in SATB1-null mice and the strong binding affinity of SATB1 for base-unpairing regions (BURs) leads

to the hypothesis that SATB1 functions to provide assembly sites for chromatin remodeling to orchestrate gene expression during thymic development.

The IL-2Rα gene is ectopically transcribed in SATB1-null thymocytes, although the SATB1-binding site is located 6.8 to 7.5 kb downstream of the transcriptional start site of this gene. Chromatin immunoprecipitation (ChIP) and micrococcal nuclease digestion of the IL-2Rα gene locus showed that SATB1 can regulate nucleosome positioning and acetylation of histones as far as 7 kb from its binding sites (218).

1.3.5 SATB1 as a transcription regulator

SATB1 has a dense three-dimensional cage-like distribution even after high salt extraction and DNase I digestion of thymocytes. This cage-like SATB1 distribution, which is referred to as a SATB1 network, plays an active role in chromatin alterations of the *myc* locus and in *myc* gene expression in thymocytes. The chromatin regions in thymocytes that bind to the SATB1 network were cloned. Genes within 50 kb of these regions were regulated by tethering to the SATB1 network. In addition, a region of the γ-globin locus associates with the nuclear matrix. Two sites within the element bind to SATB1, implying that this region may affect gene expression by nuclear matrix binding (48). However, tethering of genes to the SATB1 network and orchestrated histone acetylation and methylation can result in repression, activation, or induction, depending on the locus (30).

The DNase I-hypersensitive site (HS1) in the T-cell receptor (TCR) α locus is induced during differentiation of CD4-CD8- to CD4+CD8+ thymocytes. This HS1 region, which binds both SATB1 and CDP, associates with the nuclear matrix *in vitro*.

When the HS1 region was linked with a reporter gene along with the enhancer sequence E_β and transiently transfected into four thymoma lines, reporter gene expression was suppressed 2.5-fold compared to that of the reporter gene containing only E_β. This result implies that SATB1 acts as a repressor of TCR transcription (38).

Various MAR sequences were linked with a reporter gene and stably transfected into BHK cells, which contain concatemerized 25-bp repeats $[25]_7$ of the core unwinding region, a 2.2 kb MAR fragment from upstream of the human interferon-beta (hnIFN-β), and a 1.8 kb MAR from upstream of the potato ST-LS1 gene. Overexpressed SATB1 dramatically suppressed expression of the reporter genes linked to the MAR sequences (111). Furthermore, SATB1 has at least 7 binding sites within the $gp91^{phox}$ promoter. A strong SATB1-binding sequence and a binding mutant were each linked with a reporter gene and transiently transfected into human erythroleukemia (HEL) cells. The reporter gene expression was down-regulated by the wild-type, but not the mutant SATB1-binding sequence (80).

As suggested by experiments with SATB1-null mice, this transcription factor also may function as an activator. SATB1 binding as well as DNase I hypersensitivity and nuclear matrix-binding assays were used to identify regulatory regions of the CD8 gene (104). SATB1 binds to a MAR element (L2a) in an upstream regulatory region of the mouse CD8α gene (10), where it displaces the CDP repressor (10). The gamma-globin promoter and enhancer binding factor (γPE) is a large nuclear complex that contains SATB1 and HOXB2 proteins and binds to five sites located 5' and 3' of the human γ-globin gene. SATB1 causes an increase in the luciferase reporter activity,

whereas HOXB2 decreases reporter gene activity from the reporter gene with the γPE-binding site (32).

SATB1 also may have additional functions. The major breakpoint region (MBR) in the untranslated portion of terminal exon 3 of BCL2 is a hot spot for the t(14;18) chromosomal translocation in lymphomas, resulting in inactivation of the oncogene BCL2 and its ability to facilitate apoptosis (132,202). SATB1 was shown to bind to the 33-bp core region of the AT-rich MBR sequence by affinity purification. The MBR is a MAR sequence that contains multiple, constitutive, DNase I-hypersensitive sites of a SATB1-binding site. SATB1 binding to the MBR may enhance the intrinsic fragility of the matrix attachment region (172).

1.3.6 Role of SATB1 in apoptosis

SATB1 reportedly is specifically cleaved by caspases during apoptosis, but not during necrosis. The cleavage site of SATB1 has been mapped to the N-terminal region just before the MAR-binding domain. This site contains the VEMD sequence, which matches the cleavage motif for caspase-6, VEXD (77). However, a conflicting report showed that cleaved SATB1 fragments of 70 kD, a size similar to that of the apoptotic signature fragment, are detected during necrosis (23) and SATB1 in the nuclear matrix remains uncleaved in apoptotic HL-60 cells (130). These contrasting conclusions may result from degradation of different sets of nuclear matrix proteins during apoptosis, depending on the effectors and/or cell lines. Alternatively, SATB1 might be degraded during apoptosis, but the degradation product does not remain in

the nuclear matrix after cleavage. Therefore, the cleaved fragments would not be detected in the nuclear matrix of apoptotic HL-60 cells (130).

The site-specific cleavage of SATB1 during apoptosis initiates DNA degradation at the SATB1-binding site. A number of genes, including the c-*myc* proto-oncogene and high-mobility group phosphoprotein (HMG), were selected for analysis due to their high similarity to the 25-bp BUR element of the IgH gene that is known to contain a SATB1-binding site. Subsequently, ligation-mediated PCR (LM-PCR) revealed an increased accumulation of single-stranded DNA breaks in close proximity to these regions relative to adjacent stretches of DNA (125).

1.3.7 SATB1-binding sequences

Genomic SATB1-binding sequences *in vivo* have been isolated by immunoprecipitation of Jurkat T-cell lysates with SATB1-specific antibody on crosslinked and *Sau*3AI-digested chromatin. Cloning and sequencing revealed 16 SATB1-binding sequences (SBSs), and three were chosen as DNA probes for *in situ* hybridization experiments. These experiments showed that the SBSs remained bound to the nuclear matrixes after extensive *in situ* nuclear matrix preparation using washes with 2M NaCl and nuclease digestion. These results suggested that SATB1 binds to the bases of chromatin loops. *In situ* hybridizaion with a SBS in a human breast cancer cell line (SK-BR-3), which does not express SATB1, gave undetectable signal in the nuclear matrix (31,50), suggesting that chromatin anchoring to the nuclear matrix and loop formation also is cell-type dependent. This higher order chromatin organization by SATB1 may regulate tissue-specific gene expression.

Nuclear matrix proteins isolated from breast tumor tissues specifically bound to the MAR of the c-*erbB*-2 promoter region, but these proteins were not found in nuclear matrix from normal breast tissues. The MAR-binding activity of SATB1 was detected only in human breast carcinomas, but not in normal breast tissue, suggesting that SATB1 plays an important role in breast cancer progression (215).

1.3.8 Role of SATB1 in MMTV transcription

Deletion analysis of the MMTV LTR localized the negative regulatory elements (NREs) to the MMTV U3 region. Two regions, one between -484 and -424 and the other between -281 and -255, were shown to be important for NRE function on basal transcription from the MMTV LTR. Furthermore, two protein complexes, referred to as upper binding protein (UBP) and NRE-binding protein (NBP) specifically bound to the 100-bp fragment in the NRE. The NBP complex had a cell-type specific distribution since it was detectable in extracts from T cells, but not mammary cell (25). Purification and mass spectrometry revealed that a major component of NBP was SATB1.

SATB1 binding was shown to have a dramatic effect on tissue-specific expression from the MMTV LTR. A mutant-LTR plasmid (p924) with a 7-bp substitution mutation the SATB1-binding site of the proximal NRE had a 2.6-fold elevation of basal LTR transcription over that of plasmids with the wild-type MMTV LTR. Furthermore, transgenic mice carrying an MMTV LTR with the mutation exhibited a dramatic alteration in their expression pattern (124). Lymphoid tissues, such as the spleen, thymus, Peyer's patches, and lymph nodes of the transgenic mice with the 924 mutation showed 10 to 100-fold higher expression of the transgene when compared to

other organs, including lung, kidney, and mammary gland tissues. In contrast, mice expressing wild-type MMTV LTR-reporter genes showed the highest expression in lactating mammary tissues, and lower expression in lung, kidney, and lymphoid tissues. Since SATB1 binds to a MAR within the negative elements, these results are consistent with SATB1-mediated repression of MMTV expression in several cell types, including T cells.

1.4. RATIONALE FOR THIS STUDY

MMTV causes mammary adenocarcinomas in susceptible mouse strains. Milk-borne or exogenous MMTV infects lymphoid cells that deliver the virus to mammary epithelial cells, the final destination of the virus (57). Despite the fact that lymphoid cells are infected, wild-type MMTV does not cause lymphomas. MMTV variants that cause thymic lymphomas contain a 300- to 500-bp deletion in the U3 region of the LTR. The deleted region binds to at least two cellular proteins, CDP and SATB1 (124). SATB1 is believed to be the primary regulator of MMTV expression in T cells (53) because of its abundant expression in thymocytes and T-cell lines (124,184). Although experiments in transgenic mice and in transient assays with mutant LTRs suggest that SATB1 is a repressor of MMTV expression (25,124), a direct relationship between SATB1 and MMTV transcription has not been demonstrated. Furthermore, the mechanism by which SATB1 suppresses MMTV expression is not clear.

In this study, SATB1 domains necessary for transcriptional suppression of the MMTV LTR from integrated templates were elucidated. These experiments should lead to a better understanding of tissue-specific MMTV expression.

2. MATERIALS AND METHODS

2.1. PLASMIDS

Wild-type and mutant SATB1 cDNAs were cloned into the pUHD10-3 vector and overexpressed in MCF-7 cells stably transfected with the transactivator [MCF-7/Tet-off (Clontech)] or in cell lines expressing the transactivator plasmid, pUHD15-1. The UHD vectors were provided by Dr. Paul Bates (University of Pennsylvania School of Medicine). Full-length mouse SATB1 cDNA from pMAT (provided by Dr. Kohwi-Shigematsu) was inserted into the *Eco*RI site of pUHD10-3 to make pUHD10-3/SATB1. For the SATB1(1-345) and SATB1(1-494) constructs, full-length SATB1 cDNA was digested with *Sca*I or *Ase*I, respectively, and treated with Klenow enzyme. Each fragment was then inserted into the *Eco*RI site of pUHD10-3. Full-length SATB1 cDNA was digested with both *Sca*I and *Ase*I, treated with mung bean nuclease, and ligated. This fragment was inserted into the Klenow-treated *Eco*RI site of pUHD10-3 to make ID(346-494). pUHD10-3/SATB1 was prepared by digestion with *Eco*NI, treated with mung bean nuclease, and sequentially ligated to make ID(274-410). The pUHD10-3/SATB1 plasmid was digested with *Eco*NI and *Bam*HI, treated with Klenow enzyme, and self-ligated to construct SATB1(1-274). The plasmids SATB1(1-224), SATB1(55-764), SATB1(168-764), and SATB1(275-764) were created by PCR with primer pairs: SATB199EGFP and SA870DO, SA631UP and SA2497DO, SA700UP and SA2497DO, and SA1021AUG and SA2497DO, respectively. The PCR products were digested with *Eco*RI and inserted into the *Eco*RI site of pUHD10-3. The construct ID(224-278)

was prepared by recombinant PCR with 4 primers: SA196+, SA867-, SA1033+ and SA2497DO. Briefly, the recombinant PCR was accomplished using two rounds of PCR as previously described (67). Two fragments were separately amplified using SA196+ and SA867- primers or SA1033+ and SA2497DO primers with pMAT as the template. After combining the two PCR products, a third round of PCR was performed with SA196+ and SA2497DO primers.

For construction of pEGFPC1-NMTS and pLexA-NMTS, the SATB1 NMTS was amplified by PCR with Sagfp865+ and SA1032- or Sagal865+ and SA1032- primers. The products were cloned into the *Eco*R1 site of pEGFPC1 (Clontech) or pLexA (generously provided by Dr. Paul Gottlieb at the University of Texas at Austin), respectively. To prepare pGFP-SATB1, pGFP-SATB1(1-55), and pGFP-SATB1(1-224), fragments were amplified by PCR using primer pairs SATB199EGFP and SA2497DO, SATB199EGFP and SA363DO, and SATB199EGFP and SA870DO, respectively. The products were cloned into the *Eco*R1 site of pEGFPC1 (Clontech). The plasmid pGBKT7-NMTS was constructed by PCR amplification of the SATB1 NMTS with primers Sagal865+ and SA1032 and insertion into the *Eco*RI site of pGBKT7.

Myc-tagged lamin A (pCI-mycLMNA), lamin C (pCI-mycLMNC), and pCI-LMNB vectors were generously provided by Dr. Sue Shackleton (University of Leicester, UK). Incorporation of the Myc-tag in pCI-LMNB was accomplished by swapping the lamin B fragment obtained by digestion with *Sal*I and *Eco*RI with fragments obtained using MycLamin369+ and Lamin802-.

Primers SA196+ and SA2497DO and SA196+ and SA1686-, respectively, and pMAT were used as template for PCR, and the products were inserted into the *Eco*RI site of MigR1 to produce retroviral expression vectors MigR1-SATB1 and MigR1-SATB1(1-494). ID(346-494), ID(224-278) [also called ΔNMTS] and SATB1(168-764) fragments were digested from ID(346-494), ID(224-278), and SATB1(168-764), respectively, and cloned into the *Eco*RI site of MigR1, resulting in constructs now referred to as MigR1-ID(346-494) (also called MigR1-ΔDBD), MigR1-ID(224-278) (also called MigR1-ΔNMTS) and MigR1-SATB1(168-764), respectively.

For high fidelity, *Pfu* Turbo DNA polymerase (Stratagene) was used for all PCRs as recommended by the manufacturer, except in the 2nd round of recombinant PCR for ID(224-278), where Platinum *PfX* DNA polymerase (Invitrogen) was used. The final clones were verified by sequencing or extensive restriction enzyme digestions.

pMTV-LUC/Hyg (pMTV/Luc was previously called pLC-LUC) was made by inserting the *Xho*I fragment from pUHD15-1/hygro(-) into the *Aat*II site of pLC-LUC using an *Xho*I linker.

Table 1. Primers for SATB1 mutant cloning

Name	Sequence
Lamin802-	5'-TTC GAA TTC AGT GCT GCT TCA-3'
MycLamin369+	5'-AAC CTC GAG AAT ATG GAG CAG AAA CTC ATC TCT GAA GAG GAT CTG ATG GCG ACT GCG ACC CCC GTG-3'

SA1021AUG	5’-GCG AAT TCC TGA GTA TGC CAG GAA ACA CAG CTG AG-3’
SA1032-	5’-CTG AAT TCT GTG TTT CCT GGG ACA GG-3’
SA1033+	5’-AAC AGC ACG GCT GAG CAG CCT CCA TCC-3’
SA1043DO	5’-CGG AAT TCG GCT GCT CAG CTG TGT TTC C-3’
SA1686-	5’-GCG AAT TCC TAG GCA TTA ATG TTC ATA GTA TTG-3’
SA196+	5’-CGG AAT TCA GTA TGG ATC ATT TGA ACG AG-3’
SA2497DO	5’-CGG AAT TCT CTC TCA GTC TTT CAA GTC G-3’
SA363DO	5’-GGC GAA TTC TTA CAC TCC CTG CAT CTT TCC-3’
SA631UP	5’-GCG AAT TCC TGA GTA TGG TGC CTT TAA AAC ACT CG-3’
SA700UP	5’-GCG AAT TCC TGA GTA TGA TTC AGT TAC ACA GTT GC-3’
SA867-	5’-CTG CTC AGC CGT GCT GTT CAC AAT GGA G-3’
SA870DO	5’-GGC GAA TTC TTA GTA CGT GCT GTT CAC AAT G-3’
Sagal865+	5’-CGG AAT TCA CGT ACT ATG CAA ATG TC-3’
Sagfp865+	5’-CGG AAT TCT ACG TAC TAT GCA AAT GTC-3’
SATB199EGFP	5’-CGG AAT TCA CTG AGT ATG GAT CAT TTG AAC-3’
SATB199UP	5’-CGG AAT TCT TAT GGA TCA TTT GAA CGA GGC-3’

2.2. PLASMID DNA PREPARATION

Luria-Bertani (LB) medium containing specific antibiotics was inoculated with cells from a single colony and incubated for 6 to 8 hr. This culture was then added to 500 ml of fresh LB medium and incubated overnight. Alternatively, the plasmid DNA was amplified using 170 μg/ml chloramphenicol. The bacteria were pelleted by

centrifugation at 7,000 rpm for 10 min at 4°C. The cell pellet was suspended in Solution I (25 mM Tris-HCl, pH 8.0, 50 mM glucose, and 10 mM EDTA) supplemented with 10 mg lysozyme, and then lysed in Solution II (0.2 N NaOH and 1% SDS). The chromosomal DNA, high-molecular-weight RNA, and potassium/SDS/protein-membrane complexes were precipitated with Solution III (3 M potassium acetate, pH 5.5). The plasmid DNA was pelleted with isopropanol and washed with 70% ethanol. After dissolving the plasmid pellet in 3.5 ml TE 10:10 (10 mM Tris-HCl, pH 7.4 and 10 mM EDTA), 4.6 g CsCl and 0.68 ml ethidium bromide (5 mg/ml) were added to the solution. Ethidium bromide and the protein complex were removed, and the supernatant was subjected to ultracentrifugation at 50,000 rpm (227,000 Xg) at 20 °C for 16 hr. Ethidium bromide was extracted from purified plasmid DNA using n-butanol saturated with G-50 buffer (10 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 2 mM EDTA). The plasmid was reprecipitated with ethanol, washed with 70% ethanol, and resuspended in TE 10:10. Plasmid DNA was treated with 100 μg/ml RNase A, 200U RNase T1, and 100 μg/ml proteinase K to remove contaminating RNA and proteins. The solution was dialyzed against TE 10:1 (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA). The DNA was precipitated and dissolved in TE 10:0.1 (10 mM Tris-HCl, pH 7.4 and 0.1 mM EDTA).

2.3. CELL CULTURE

Human 293T kidney cells, BW5147 mouse T cells, and MCF-7/Tet-off human breast cancer cells (hereafter called MCF-7) (Clontech) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 Units penicillin and 100 μg/ml streptomycin (Gibco), 2 mM L-glutamine (Gibco), and 50

μg/ml gentamicin. The media for MCF-7 cells was supplemented with 100 μg/ml Geneticin (Gibco) to maintain the transactivator plasmid as recommended by the manufacturer. XC rat fibroblast cells were grown in the same media as that described for 293T and MCF-7 cells, except that 5% FCS was used. C3H/XC cells were made by Dr. Jaquelin Dudley by infecting XC cells with the C3H strain of MMTV. Jurkat human T cells were grown in Roswell Park Memorial Institute (RPMI) medium supplemented with the same additives as 293T cells. HC11 cells were grown in RPMI supplemented with 10% FCS, 2 mM L-glutamine, 50 μg/ml gentamicin, 10 μg/ml insulin, and 10 μg/ml epidermal growth factor (EGF) (Invitrogen). HC11/MTV-LUC cells and XC/MTV-LUC cells kindly were provided by Dr. Keqin Gregg and Dr. Quan Zhu, respectively. Stably transfected XC/MTV-LUC, Jurkat/MTV-LUC, BW5147/MTV-LUC and HC11/MTV-LUC cells were grown in the same medium as the parental cells with the addition of 250 μg/ml hygromycin B (Invitrogen). All the cell lines were maintained at 37°C in an atmosphere of 7.5% CO_2.

To suppress SATB1 expression in MCF-7/Tet-off cells during the selection, 1 μg/ml doxycycline (DOX) was used for supplementation of the growth medium. The media was changed every 3 days. SATB1 expression in the stably transfected MCF-7/SATB1 cells was achieved by using medium without DOX. Dexamethasone (DEX) (10^{-6}M) was used in growth medium to induce MMTV expression 24 hr prior to extraction of RNA or protein from XC/MMTV, XC/MTV-LUC, HC11/MTV-LUC, and BW5147/MTV-LUC cells.

2.4. TRANSFECTIONS

For subcellular fractionation and nuclear extracts, XC and MCF-7 cells were plated at densities of 7.5 X 10^5 and 4 X 10^6 cells per 60-mm-diameter plate, respectively, for 24 hr prior to the transfection procedure. DMRIE-C reagent (Invitrogen) or SuperFect (Qiagen) was used as recommended by the manufacturer to introduce the SATB1 (6 μg) and pUHD15-1/hygro (4 μg) constructs into XC cells. For MCF-7 cells, DMRIE-C was used with 6μg of SATB1 construct. A total of less than 5 μg of DNA was used with SuperFect as a transfection reagent.

For reporter gene assays, MCF-7 cells were transfected with 40 μg DNA and 1X10^7 cells in 200 μl complete media in a 2mm cuvette (BTX) using a BTX electroporator at settings of 150V, R4, and 1750 μF. The transfected cells were incubated for 24 to 48 hr prior to preparation of extracts.

To prepare BW5147/MTV-LUC, BW5147 cells were electroporated with 40 μg pMTV-LUC/Hyg and 1.5 X 10^7 cells in 300 μl complete media in a 4mm cuvette (Bio-Rad) using a Bio-Rad electroporator at settings of: high range (700), high capacitance (500 V max), voltage 0.3, and capacitance 0.950. The transfected cells were selected with 500 μg/ml hygromycin B (Invitrogen) for 3 weeks.

Jurkat/MTV-LUC cells were prepared by electroporation of Jurkat cells with 40 μg pMTV-LUC/Hyg and 6 X 10^6 cells in 400 μl complete media in a 4mm cuvette (BTX) using an Electro cell manipulator (BTX) at settings of 1050 μF, R10, and 260V. The transfected cells were selected with 250 μg/ml hygromycin B (Invitrogen) for 3 weeks.

2.5. SUBCELLULAR FRACTIONATION

Subcellular fractions were prepared according to the method developed by de Belle et al. (50) with slight modifications. MCF-7 cells (4 X 10^6) were transfected with various pUHD10-3/SATB1 constructs and incubated for 24 to 48 hr. The cells were incubated for 4 min in 100 μl of 0.5% Triton X-100 in CSK buffer (10 mM PIPES, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, and 1mM EGTA) supplemented with 1 mM PMSF, and 1X protein inhibitor cocktail (AEBSF, bestatin, E-64, Leupeptin, Pepstatin A, and 1,10-phenanthroline) (Sigma) on ice. Following centrifugation (6,000 Xg), the supernatant was removed, and designated as the soluble fraction. The insoluble fraction was suspended in 50 μl CSK buffer and treated with 20 units of DNase I (Roche) for 15 min at 37°C, followed by ammonium sulfate precipitation at a final concentration of 0.25 M. The insoluble material was removed by centrifugation at 6,000 Xg, and the soluble material was designated as the chromatin fraction. The insoluble material was washed with 2 M NaCl in CSK buffer, dissolved in 8 M urea buffer (8 M urea in 10 mM Tris-HCl, pH 8.0), and designated the nuclear matrix fraction. The same ratio of each fraction was used for analysis on 8 to 15% SDS-containing polyacrylamide gels, depending on the size of the SATB1 mutant.

2.6. YEAST TWO-HYBRID SCREEN

Yeast two-hybrid screening was performed according to the supplier (Clontech). Briefly, the SATB1 NMTS was used as bait to screen a human thymus Matchmaker cDNA library (Clontech). The bait and the amplified library were sequentially transformed into the yeast strain AH109 using the lithium acetate method. The

transformants were assayed for their expression of histidine (*HIS3*) reporter gene on plates containing selective dropout (SD) media without histidine, tryptophan, or leucine. Colonies that grew on the selective medium were replated on the same selective medium plates twice to eliminate false-positive clones. To enrich for the weakly interacting clones, the screening was performed in two steps. First, all of the transformants were plated on SD plates lacking tryptophan and leucine to select transformants with both pGBKT7-NMTS and pACT2-Library plasmids. The selected transformants then were scraped from the plates, and individual colonies were plated on SD medium lacking histidine, tryptophan, and leucine and on SD medium lacking histidine, adenine, tryptophan and leucine to select clones interacting with the SATB1 NMTS. Positive clones were sequenced, and the interaction was further verified by other biochemical methods.

2.7. IMMUNOPRECIPITATION

The 293T cells in 100-mm plates were transfected with expression vectors as described above. After 24 to 48 hr, cells were collected by centrifugation and washed once with PBS. Protein extracts were obtained by incubation of cell pellets for 5 min on a rotating wheel with 600 ul of lysis buffer (50 mM HEPES, pH 7.9, 150 mM NaCl, 1% Trition X-100, and 10% glycerol supplemented with 1 mM PMSF and protease inhibitor cocktail (Sigma; P-9599). The cell debris was then removed by centrifugation (15,000 Xg), and the remaining supernatant was pre-cleared with protein A/G plus agarose (Santa Cruz Biotechnology) at 4^{o}C followed by centrifugation at 10,000 Xg for 20 sec. The supernatant was incubated at 4^{o}C for 2 hr with Myc-specific (Babco; 1:100

dilution), GFP-specific (BD Biosciences; 1:100 dilution), or p300-specific (Santa Cruz Biotechnology; 1:50 dilution) antibodies. Protein A/G plus agarose beads were added, incubated for an additional hour, and washed three times with lysis buffer. Samples were solubilized in 2X SDS-loading buffer (1X = 0.06 M Tris-HCl, pH 6.8, 1.7% SDS, 6% glycerol, 0.1 M DTT, 0.002% bromophenol blue), boiled, and separated on 8 to 10% polyacrylamide gels containing SDS.

2.8. WESTERN BLOTTING AND ANTIBODIES

Protein samples were mixed with 6X SDS-loading buffer, boiled, and separated on 8 to 15% polyacrylamide denaturing gels. The proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) at a constant current of 150 mA overnight at 4 ^{o}C with transfer buffer (47.8 mM Tris-HCl, pH 8.3, 0.39 M glycine, 0.1% SDS and 20% methanol). The membrane was blocked with TBST buffer (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20) containing 5% milk and incubated with primary antibodies in 1% milk in TBST buffer. The membrane was washed three times with TBST buffer and detected by horseradish peroxidase-conjugated antibodies that were specific for the primary antibody. Enhanced chemiluminescence reagents (NEN) were used for detection. The following antibodies were used: mouse monoclonal SATB1 (BD Biosciences; 1:2,000 dilution), goat polyclonal GFP (Clontech; 1:1,000 dilution), mouse monoclonal LexA (Clontech; 1:1,000 dilution), mouse monoclonal Myc (Babco; 1:1,000 dilution). All secondary antibodies (mouse, rabbit, goat immunoglobulin (Ig)-specific antibodies) conjugated to horseradish peroxidase were purchased from Jackson Immunoresearch. p300-specific antibody (Santa Cruz;

1:200 dilution), Lamin B-specific antibody (Santa Cruz; 1:200 dilution), and mouse, goat, and rabbit normal sera (Santa Cruz; immunoprecipitation controls) were also used. MMTV CA (p27)-specific mouse monoclonal antibodies (dilution 1:50) were generously provided by Dr. Tatyana V. Golovkina (The Jackson Laboratory) (167). Rabbit polyclonal SATB1- and CDP-specific antibodies have been previously described (124). Rabbit polyclonal SATB1-specific antibodies also were provided by Dr. Paul Gottlieb (The University of Texas at Austin) (dilution 1:1,000). All antibodies were diluted in PBS.

For quantitation, Western blots were incubated with GFP-specific or lamin B-specific antibodies, washed, and incubated with Alexa Fluor 680-conjugated rabbit Ig-specific (Molecular Probes) or IRDye800-conjugated goat Ig-specific (Rockland) antibodies. Band intensities were determined using infrared fluorescence and an Odyssey imaging system (LI-COR Biotechnology). Blocking buffer (LI-COR) was used for pretreatment of blots and antibody dilution.

2.9. VIRUS PRODUCTION, CONCENTRATION, AND TITERING

The day prior to transfection, 293T cells (8 X 10^6) were plated on a 100-mm dish. Cloned MigR1 plasmid, pHIT60 and pHITG were co-transfected at a 2:1:1 ratio (total DNA was 8 μg) using SuperFect (Qiagen) according to the manufacturer's directions. The transfection complexes were incubated for 18 to 20 hr, and then fresh medium supplemented with 10 mM n-butyric acid (3 ml) was added to induce virus production for 7 hr. After 24 hr of incubation, the supernatant containing virus particles was recovered. Cell debris was removed by centrifugation at 1,500 rpm (450 Xg).

Virus particles remaining in the supernatant were pelleted by ultracentrifugation for 100 min in a SW55Ti rotor (Beckman) at 20,000 rpm (38,000 Xg). The viral pellet was resuspended in complete media, aliquoted and frozen at -70 ^{o}C until needed. To determine the titer, 5 and 10 μl of viral stock were incubated with 1 X 10^5 Jurkat cells to obtain the linear range of infection. The infection efficiency was determined by fluorescence-activated cell sorter (FACS) analysis at 24 to 48 hr post-infection using the fluorescence from the expressed *gfp* gene in the MigRI vector (see below).

2.10. TOTAL RNA EXTRACTIONS AND RNase PROTECTION ASSAYS (RPAs)

Total RNA was extracted using the guanidinium thiocyanate method with slight modifications (41). Briefly, 1 ml of Solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% Sarcosyl, and 0.1 M 2-mercaptoethanol), 0.1 ml 2M sodium acetate, pH 4.0, and 1 ml water-saturated phenol were directly added to cells on a 100-mm dish. The phenol-treated cell mixtures were transferred into microcentrifuge tubes and incubated with 0.2 ml of chloroform for 15 min on ice. After centrifugation, the aqueous phase was mixed with the same volume of isopropanol to precipitate RNA. The RNA pellet was washed with 70% ethanol and dissolved in water.

RNase protection assays were performed essentially as described by Wrona *et. al.* (212) except that hybridizations were performed at 55^{o}C. C3H riboprobe template (pTSau3A) was constructed by inserting the C3H LTR sequence from +843 to +1086 into the pGEMT-Easy vector (Fisher Scientific). The pTSau3A plasmid was digested with *Nde*I, and labeled RNA probe was produced using T7 polymerase. Mouse GAPDH and cyclophilin plasmids (Ambion) were used for RNA internal controls as

recommended by the manufacturer. Briefly, C3H and internal control probes were labeled with α-^{32}P-UTP by *in vitro* transcription. The labeled probes were hybridized with total RNA by overnight incubation at 55^{o}C in hybridization buffer (80% deionized formamide, 400 mM NaCl, 40 mM PIPES, pH 6.4, and 1mM EDTA). The unhybridized RNAs were digested with RNase A and RNase T1 in RNase digestion buffer (300 mM NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA) at 37 ^{o}C for 3 hr followed by digestion with 130 µg/ml proteinase K for 1 hr at 37 ^{o}C. The RNA was precipitated with ethanol, resuspended, and fractionated on a 6% sequencing gel containing 7M urea. The gel was dried and subjected to autoradiography. To quantitate the amount of both C3H MMTV and the internal control RNAs, the dried gel was exposed on a phosphoimager screen (Molecular Dynamics), and the image analysis was performed using ImageQuant software.

2.11. REVERSE TRANSCRIPTASE (RT)- PCR

Total RNA (20 µg) was treated with DNase I (3U, Invitrogen) for 1 hr at 37 ^{o}C to digest contaminating DNA. To stop the reaction, EDTA was added to 0.5 mM, and the mixture was heated for 10 min at 70^{o}C. The DNase-treated RNA was adjusted to 0.58 mM deoxynucleodtides and 50 pmol oligo(dT)$_{17}$ (IDT) and boiled for 5 min. Murine leukemia virus RT (500 U, Invitrogen) was added and incubated for 1 hr at 37^{o}C. The cDNA was then used with appropriate primer sets for PCR analysis.

2.12. *IN SITU* NUCLEAR MATRIX INTERMEDIATE FILAMENT (NMIF) PREPARATION

Cells grown on cover glasses were extracted stepwise to prepare nuclear matrix intermediate filament (NMIF) according to the method of Javed *et. al.* (97) with slight modifications. Briefly, cells were extracted twice for 15 min with ice-cold CSK buffer. The chromatin was then digested by two consecutive 30 min incubations at 25°C with 50 U RNase-free DNase I (Roche Molecular Biochemicals) in DB buffer (CSK buffer with 50 mM NaCl). The NMIF was obtained by further extraction for 10 min on ice with stop solution (DB with 0.25 M ammonium sulfate).

2.13. INDIRECT IMMUNOFLUORESCENCE MICROSCOPY

Cells grown on cover glasses were washed with PBS and fixed by treatment with 4% ice-cold paraformaldehyde in PBS for 15 min at room temperature. The cells were washed with PBS three times and permeabilized with 0.1% Triton X-100 in PBS for 10 min. Again, the cells were washed with PBS three times. A primary antibody was diluted in antibody dilution solution (ADS:10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2% FBS, and 0.1% Tween-20) and incubated with the cells on the coverslip for 30 min at 37°C. If the cells were stained with more than one primary antibody, the second primary antibody was added after PBS washing and incubated for another 30 min at 37°C. Following the final PBS wash, appropriate FITC- or rhodamine- conjugated secondary antibodies (Santa Cruz) were incubated with cells for 30 min at 37°C. The stained cells were washed with PBS 3 times and mounted on slides with Vectashield (Vector Laboratories) containing 300 nM DAPI to visualize the nucleus. The images

were observed with confocal (Leica TCS 4D) or fluorescence microscopes (Zeiss Axioskop).

2.14. REPORTER GENE ASSAYS

Cells transfected with pMTV-LUC plasmid were collected by centrifugation and washed with PBS. Cell pellets were resuspended in 1X passive lysis buffer (Promega) and lysed by two freeze/thaw cycles using a dry ice-ethanol bath and a 37°C waterbath. The protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad), and similar amounts of protein were used to determine the luciferase activity. Luciferase assays were performed according to the manufacturer's recommendation (Promega; E1980). The final luciferase activity was normalized to a constant amount of protein for each cell lysate. A Turner TD-20e luminometer was used to measure luciferase activity.

2.15. KNOCK-DOWN OF GENE EXPRESSION USING SMALL INTERFERING (si) RNAs

SATB1-specific small interfering RNAs (siRNAs) (Dharmacon, SMART pool siRNA) or a non-targeting siRNA (Dharmacon, siCONTROL Non-Targeting siRNA #1) were introduced by electroporation. Briefly, the day prior to transfection, Jurkat/MTV-LUC cells were diluted two-fold. Cells (6×10^6) in 400 µl were mixed with the siRNA (final concentration of 600 nM) in a 4-mm cuvette (BTX), incubated for 10 min at 25°C, and electroporated using the following parameters (1050 µF, R10, and 260V) with an Electro cell manipulator (BTX). For mock transfections, all procedures were performed

without siRNA. The cells were collected between 2 to 3 days after transfection, and extracts were prepared for Western blot analysis and luciferase assays.

2.16. NUCLEAR EXTRACT PREPARATION

A nuclear extraction method (8) was performed on ice or at 4^{o}C in a cold room. Approximately 8 X 10^{8} cells (40 confluent 100-mm plates) were washed with PBS, centrifuged, and resuspended in ice-cold buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.5 mM PMSF). The cells were lysed with a Dounce homogenizer using a type B-pestle, and the nuclei were collected by centrifugation at 800 Xg for 10 min. The pellet was suspended in buffer A and subjected to centrifugation at 25,000 Xg for 20 min. The nuclear pellet was resuspended in buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT) and lysed with a Dounce homogenizer and a type B-pestle. The nuclear proteins were further extracted by stirring the lysates. The insoluble fraction was removed by centrifugation at 25,000 Xg for 30 min. The final nuclear extract was dialyzed against 50 volumes of buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF) for 4 to 5 hr. Precipitates from the dialysis were removed by centrifugation at 25,000 Xg for 30 min. The final nuclear extracts were stored at -70^{o}C until needed. However, nuclear extracts from transfected cells were prepared more rapidly using NE-PER nuclear and cytoplasmic extraction reagents (Pierce) according to the manufacturer's recommendations.

2.17. ELECTROPHORETIC MOBILITY SHIFT ASSAYS (EMSAs)

Gel mobility shift assays were performed essentially as described by Dickinson et al. (53) and Liu et al. (124). Binding reactions were performed in EMSA buffer (10 mM HEPES, pH 7.9, 50 mM KCl, 2.5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, and 1 mM PMSF) containing 0.1 mg/ml poly(dIdC) and nuclear extracts (0.5 µg) in a total volume of 10 or 15 µl. Samples were pre-incubated for 10 min on ice before ^{32}P-labeled probes (ca. 2.5 fmol) were added for another 20 min. The binding mixture was added to loading buffer (1X EMSA buffer, 80% glycerol, 0.1% xylene cyanole FF, and 0.1% bromophenol blue) and subjected to electrophoresis at 200V for 4 hr using 3.4% or 4% polyacrylamide gels. The NRE4 probe containing 4 repeats of a 25-bp strong SATB1 binding sites in the MMTV promoter-proximal NRE (124) was used for the assays. The gels were dried onto chromatography paper and exposed to X-ray film.

For antibody ablation assays, sera (1:5 dilution in PBS) were added to the binding mixture before adding the labeled probe. The antibody mixture then was incubated for 30 min on ice.

2.18. FLOW CYTOMETRY

Cells were infected with MigR1-based virus using indicated MOI (see figure legends) and expanded for 4 to 5 days. Since the MigR1 retroviral vector expresses GFP from the same mRNA as the cloned gene, the percentage of cells expressing GFP was determined by FACS and used to derive the percentage of cells expressing the cloned gene in the total population. Uninfected cells were used as the GFP-negative control. The basal level fluorescence from uninfected cells was then set as

the threshold defining GFP-positive cells. The fluorescence of infected cells was measured by the BD FACSCalibur Flow Cytometry System. Data was analyzed using CellQuest software (BD Biosciences).

3. RESULTS

3.1 DEVELOPMENT OF AN MMTV REPORTER SYSTEM FOR SATB1 FUNCTION

SATB1 is a repressor or an activator depending on the promoter context of its binding sites (5,32,66,111) . However, mutational analysis of the MMTV LTR in cell culture assays and in transgenic mice indicate that SATB1 negatively regulates MMTV expression (25,124). Deletion of SATB1-binding sites or mutation of a strong SATB1-binding site at +924 in the MMTV LTR increased reporter gene activity compared with that from the wild-type promoter in transient transfection assays (25). Transgenic mice with the +924 substitution mutation had dramatically increased reporter gene expression in the thymus relative to the mammary gland compared to that observed for animals with the wild-type MMTV LTR transgene (124). Thymocytes have high levels of SATB1 and low levels of MMTV expression, consistent with a role for SATB1 in repression of MMTV transcription (53,124). Nevertheless, a tissue culture system for analysis of SATB1 structure/function has not been established. Therefore, several assays were designed based on transfection and transduction of expression plasmids into cells with low endogenous SATB1 expression.

3.1.1 Transiently transfected MMTV reporter genes show non-specific suppression by SATB1.

To develop a tissue culture assay for SATB1 function, it would be useful to overexpress wild-type or mutant SATB1 in cell lines that lack endogenous SATB1

expression. Initially, various cell lines were analyzed by Western blot analysis to determine the endogenous levels of SATB1. A mouse mammary cell line (HC11), a rat fibroblast cell line (XC), a mouse B-cell line (A20), a mouse T-cell line (BW5147), and human breast cancer cell line (MCF-7) showed low SATB1 expression. A mink lung cell line (CCL-64) showed intermediate levels of SATB1, while a human T-cell line (Jurkat) showed the highest SATB1 expression among all the cell lines analyzed (Fig. 6).

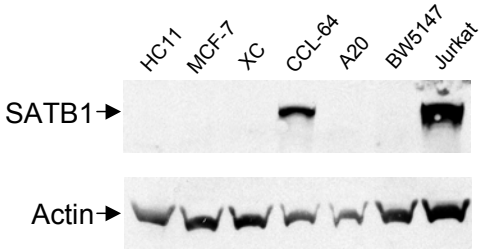


Fig. 6. Levels of SATB1 expression in various cell lines.
The level of endogenous SATB1 was measured by Western blot analysis and incubation with SATB1-specific monoclonal antibody (upper panel). Actin-specific antibody was used to ensure equal protein loading in each lane.

Several reports have indicated that SATB1 is cytotoxic in certain cell types (68,111). Therefore, an inducible gene expression system (Tet-off) was utilized to generate cell lines that stably express exogenous SATB1. The MCF-7/Tet-off (Clontech) that stably express a tetracycline-suppressible transactivator were transfected by electroporation with a pUHD10-3/SATB1 plasmid carrying a

hygromycin-resistance cassette. Following transfection, hygromycin and a tetracycline derivative, doxycycline (DOX), were added to the growth medium to select clones with integrated SATB1 transgenes and to suppress SATB1 expression during selection, respectively. Hygromycin-resistant clones were isolated, amplified, and tested for SATB1 expression by Western blot analysis. The lysate from Jurkat cells was used as a positive control. Nine clones showed detectable levels of SATB1 expression, but the expression levels were variable (Fig. 7).

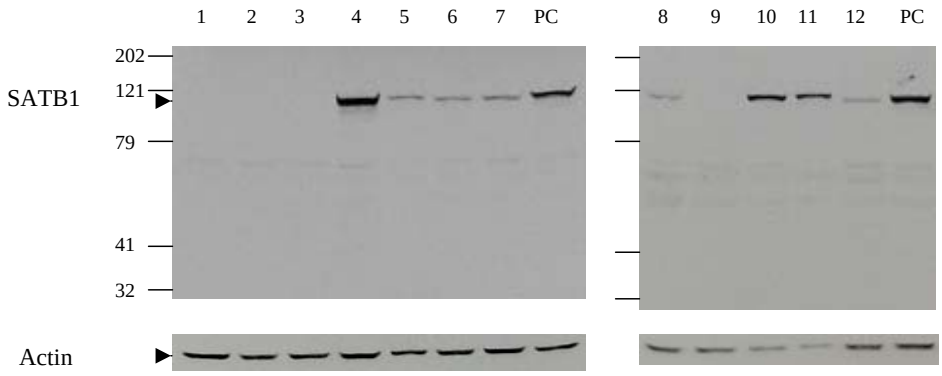


Fig. 7. Selection of MCF-7/Tet-off cells with inducible high levels of SATB1.
MCF-7/Tet-off cells were transfected with pUHD10-3/SATB1. Following transfection, DOX was added to the growth medium to suppress SATB1 expression during selection. Single colonies were isolated and expanded. Protein extracts from each clone (given by numbers above the lane) grown in the absence of DOX were prepared and used for Western blot analysis with SATB1-specific antibody (upper panel). Actin-specific antibody was used to verify equal protein loading (lower panel). Jurkat cell extracts provided a positive control (PC) for SATB1 expression.

Clones 4, 5, 6, 8, and 10 were selected and further analyzed for inducibility and effects of SATB1 expression on the MMTV LTR promoter. All the clones showed SATB1 expression in the absence of DOX. Clones 8 and 10 showed SATB1 expression in the presence of DOX. However, the overall SATB1 level was greatly increased after removal of doxycycline, even in the clones with leaky expression (Fig. 8).

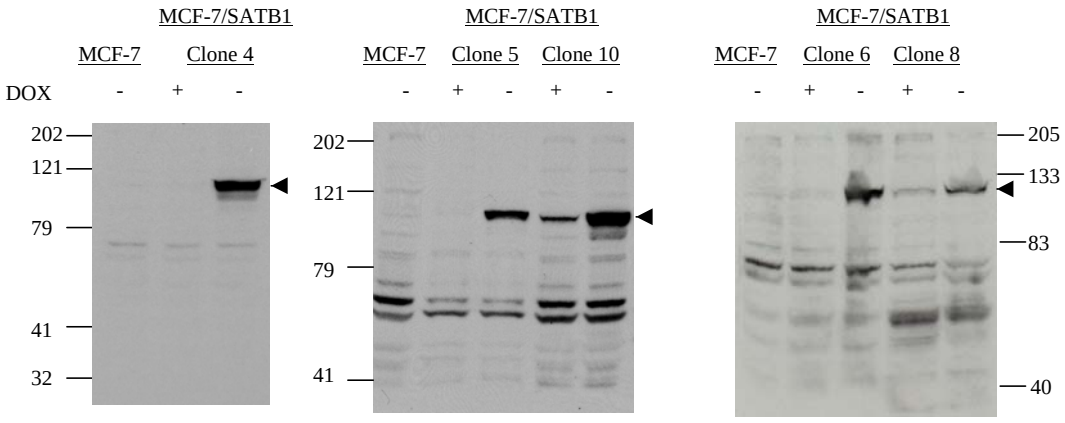


Fig. 8. SATB1 is induced in the absence of DOX in MCF-7/SATB1 clones.
Each clone was grown in the presence or absence of DOX for 48 hr. Protein extracts were prepared and analyzed by Western blotting with SATB1-specific monoclonal antibody. Arrowheads indicate the position of full-length SATB1.

To determine whether the exogenous SATB1 in MCF-7/SATB1 cells is capable of binding to the MMTV promoter, an electrophoretic mobility shift assay (EMSA) was performed using a probe derived from the promoter-proximal MMTV NRE. The probe

bound poorly to proteins in nuclear extracts from clones 4, 5, and 10 (data not shown). Therefore, a concatemer of a strong SATB1-binding site (NRE4) (124) was used as a probe to detect SATB1 binding to the MMTV NRE (Fig. 9). Binding was detected using nuclear extracts from SATB1 stably transfected cells (clone #4), but not with nuclear extracts from stable cells carrying the empty vector or in HC11 cells lacking endogenous SATB1. The band had a mobility similar to that detected using extracts from Jurkat cells, which express high levels of SATB1. Furthermore, the binding observed using extracts from MCF-7/SATB1 cells was abolished by SATB1-specific antibody, but not with CDP-specific antibody.

Unfortunately, DOX suppressed expression of a transiently transfected C3H MMTV LTR-luciferase (pMTV-LUC) reporter gene in MCF-7/Tet-off cells (Fig. 10). This effect is probably due to the overexpression of the transactivator in MCF-7/Tet-off cells since DOX did not affect MMTV transcription in untransfected XC cells (data not shown). Therefore, the effects of SATB1 were determined by comparison of reporter gene activities of cells stably transfected with empty vector or with SATB1 in the absence of DOX (Fig. 11).

To measure effects of SATB1 overexpression on MMTV transcription, MCF-7/SATB1 and MCF-7/Vector cells were transiently transfected with MMTV-LUC and assayed for luciferase activity. SATB1 overexpression was confirmed by Western blot analysis and SATB1-specific antibody. All clones except clone #10 showed suppression of luciferase activity by SATB1 overexpression (Fig. 11). Clone #10 cells had leaky SATB1 expression in the presence of DOX (Fig. 8) and, therefore, cells were exposed to the cytotoxic effects of SATB1 during the drug selection period. Surviving

cells might then be resistant to the functional effects of SATB1 overexpression. It should be noted that the clone showing the highest levels of DOX-suppressible SATB1 expression (C.4) also had the greatest effect on luciferase expression.

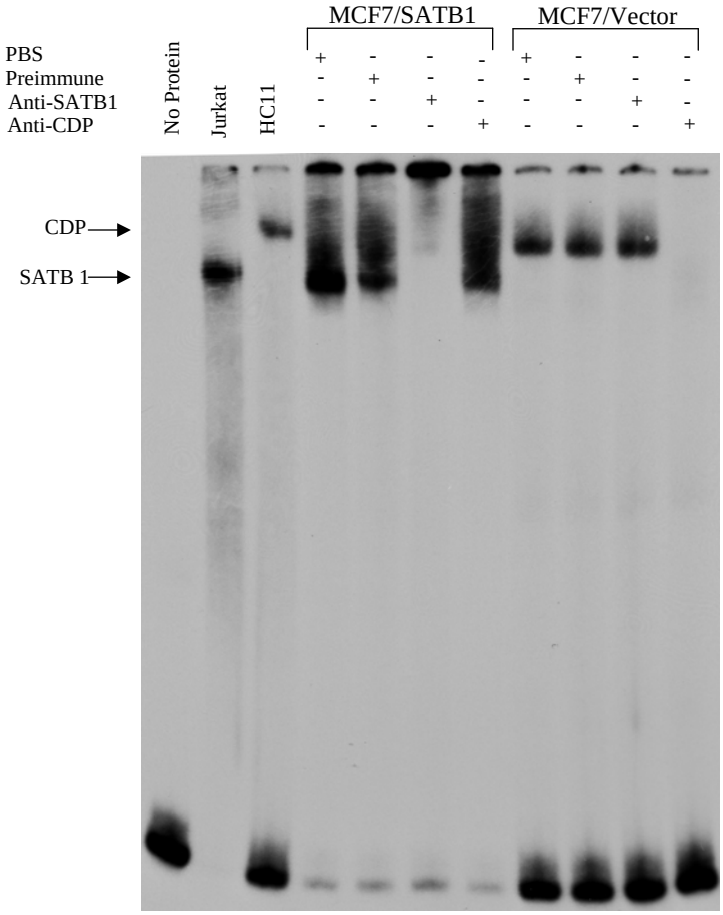


Fig. 9. SATB1 overexpressed in MCF-7/SAB1 cells binds to the NRE4 probe.

Nuclear extracts were prepared from stably SATB1-transfected clone #4 (MCF-7/SATB1) and cells stably transfected with the empty vector (MCF-7/Vector) for EMSA. NRE4, a concatemer of strong SATB1-binding sites in the NRE, was used as a probe. Nuclear extracts from Jurkat cells that express endogenous SATB1 were used as a positive control, whereas nuclear extracts from HC11 cells that do not express endogenous SATB1 were used as a negative control. The identities of the shifted bands from the MCF-7/SATB1 nuclear extracts were verified by antibody ablation assays. SATB1-specific antibody abolished SATB1 binding to the NRE4 probe, while CDP-specific antibody did not.

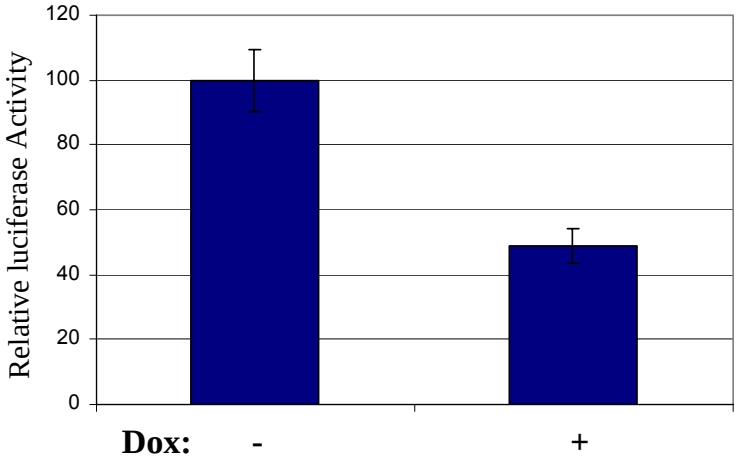


Fig. 10. DOX suppresses expression of transiently transfected MMTV-luciferase plasmids in MCF-7/Tet-off cells.

MCF-7/Tet-off cells were transiently transfected with pMTV-LUC reporter plasmid. The cells were incubated in the presence or absence of DOX for 24 hr and protein extracts were assayed for luciferase activity. The data represent means ± standard deviations of three independent transfections. The luciferase activities of samples obtained from cells grown without DOX were set to 100, and the luciferase activity of the second sample using the same amount of protein extract was determined relative to this value.

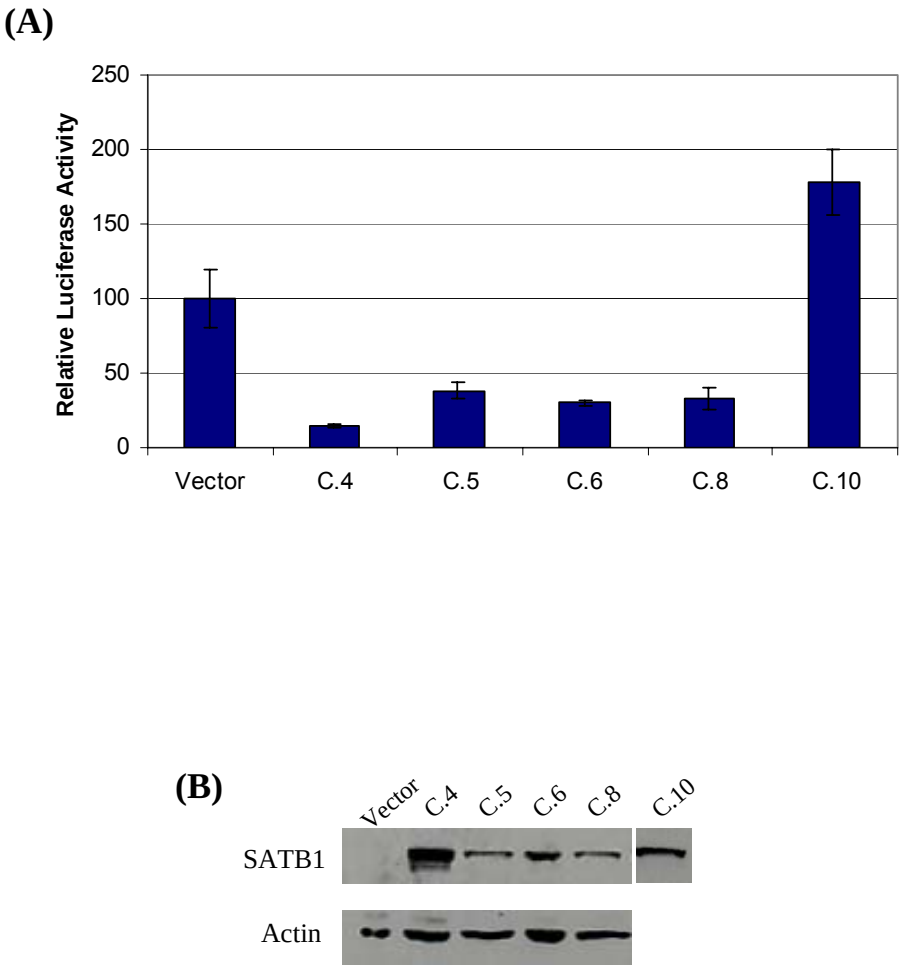


Fig. 11. SATB1 suppresses transiently transfected MMTV-reporter plasmids in MCF-7/SATB1 clones.

(A) Each clone stably transfected with SATB1 or vector was transiently transfected with a constant amount of pMTV-LUC reporter plasmid. The transfected cells were maintained in the absence of DOX for 48 hr to induce SATB1. All the SATB1-expressing clones, except clone #10 (C.10), showed decreased luciferase activity compared to a clone expressing the empty vector. The data represent the means ± standard deviations of three independent transfections. (B) Western blotting of protein extracts and incubation with SATB1 or actin-specific antibody (upper and lower panels, respectively).

The dose dependence of SATB1 effects on pMTV LTR-reporter expression was determined. Expression levels of SATB1 can be controlled in MCF-7/SATB1 cells by the DOX concentration in some clones; however, since DOX itself affected MMTV expression in these cells (Fig. 10), this method could not be used. Therefore, both the SATB1 expression construct and the pMTV-luciferase reporter plasmid were transiently transfected into MCF-7 cells. Various amounts of SATB1 expression construct were transfected with a constant amount of the pMTV-LUC plasmid in MCF-7 cells, and the empty vector was used to maintain constant levels of transfected DNA. SATB1 overexpression resulted in up to 30-fold suppression of transiently transfected MMTV-luciferase activity in a dose-dependent manner (Fig. 12A). Western blot analysis showed that SATB1 expression increased concurrently with the amount of the SATB1 expression plasmid (Fig. 12B).

The MMTV LTR promoter can be induced by binding of glucocorticoid hormone receptor complex to the hormone-responsive element (HRE) (226). Therefore, the ability of SATB1 to suppress glucocorticoid-induced pMTV transcription was determined. The SATB1 or empty expression vector was co-transfected with MMTV-LUC into MCF-7 cells by electroporation. After 24 hr, the growth medium was supplemented with the hormone dexamethasone (DEX) (10^{-6} M) for an additional 24 hr to induce luciferase expression. SATB1 suppressed reporter gene expression both in the presence and absence of glucocorticoids (Fig. 13).

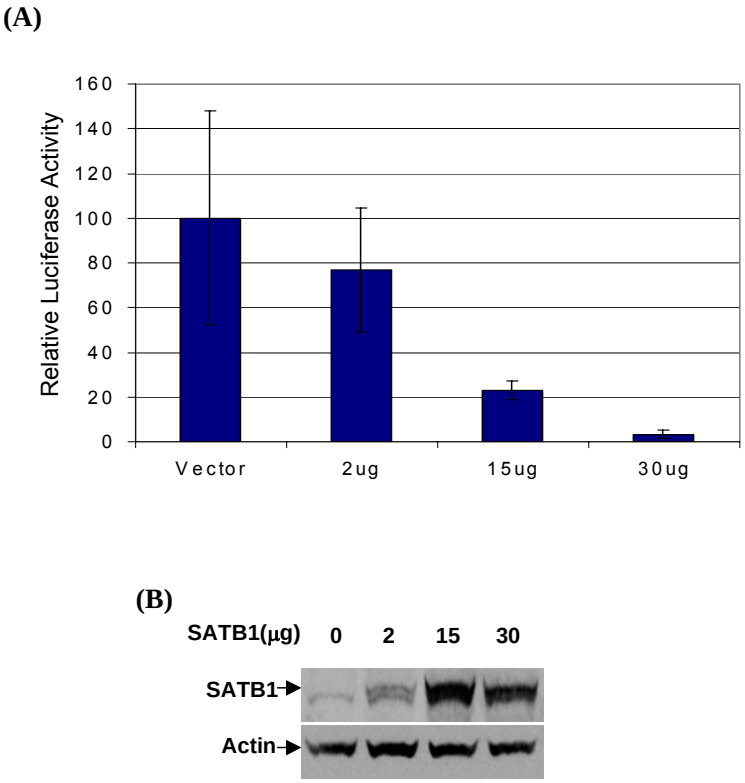


Fig. 12. SATB1 suppresses transiently transfected MMTV reporter gene expression in a dose-dependent manner.

(A) Increasing amounts of SATB1 expression plasmid (0, 2, 15, 30μg) were co-transfected in MCF-7 cells with a constant 20μg of MMTV-Luciferase reporter gene by electroporation. The empty vector was added to maintain a constant amount of total DNA. SATB1 overexpression suppressed luciferase activity 30-fold compared to the activity of empty-vector-transfected cells. The data represent means ± standard deviations of three independent transfections. (B) A Western blot using SATB1 antibody confirmed overexpression of SATB1, while actin antibody confirmed equal protein loading.

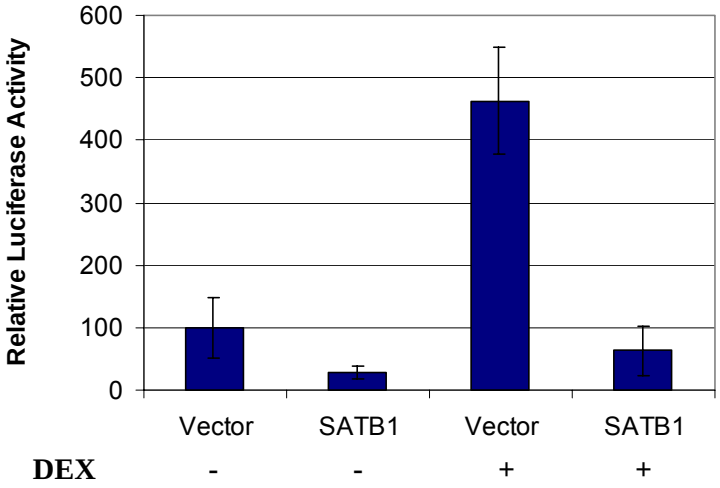


Fig. 13. SATB1 overexpression represses the luciferase activity of transiently transfected MMTV-reporter plasmids in the presence and absence of DEX.

MCF-7 cells were transiently co-transfected with SATB1 or empty vector and an MMTV-luciferase reporter plasmid and incubated for 24 hr. The transfected cells were incubated with or without DEX for an additional 24 hr, and the luciferase activity was measured. DEX increased expression from the MMTV LTR-luciferase reporter approximately 5-fold. However, SATB1 suppressed MMTV expression in the absence and presence of DEX approximately 3-fold and 7-fold, respectively.

To further study the function of SATB1 in transcriptional repression, a series of deletion mutants was constructed (Fig. 14). Four N-terminal, three internal, and four C-terminal deletions were prepared based on the known domain structure of SATB1. The SATB1 N-terminus has a proposed PDZ-like domain involved in protein dimerization (amino acids 90 to 204) as well as the recruitment of HDAC (68,218). SATB1 has several DNA-binding domains (DBDs), including two Cut repeat domains (CR1 and CR2) and an atypical homeodomain (52,149). To verify expression of all

mutant proteins, MCF-7 cells were transfected with the wild-type or mutant constructs and incubated for up to 48 hr. Whole-cell protein extracts were prepared and analyzed by Western blotting with a polyclonal antibody, which recognizes the N-terminal 10 amino acids of SATB1 (Fig. 15A), or a mouse monoclonal antibody, which recognizes the SATB1 C-terminus (amino acids 550-667) (Fig. 15B). Most constructs, except for SATB1(1-224) and SATB1(510-764), were expressed at levels comparable to wild-type SATB1.

The deletion mutants were used to determine the specificity of SATB1-mediated repression of the MMTV LTR in transient assays. The pMTV-LUC expression plasmid was co-transfected into MCF-7 mammary cells with plasmids expressing wild-type or mutant SATB1 proteins or the empty vector. As expected, wild-type SATB1 suppressed luciferase expression 10-fold. Surprisingly, all of the deletion constructs, including those missing the major DNA-binding domain (MAR domain), repressed luciferase expression from the MMTV LTR relative to that observed for the empty vector (Fig. 16). Similar results were obtained when another cell line, XC rat fibroblasts, were used (data not shown). These results suggest that DNA binding is not required for the SATB1-mediated transcriptional repression observed with unintegrated DNA templates, and that this repression is non-specific, perhaps due to squelching of general transcription factors.

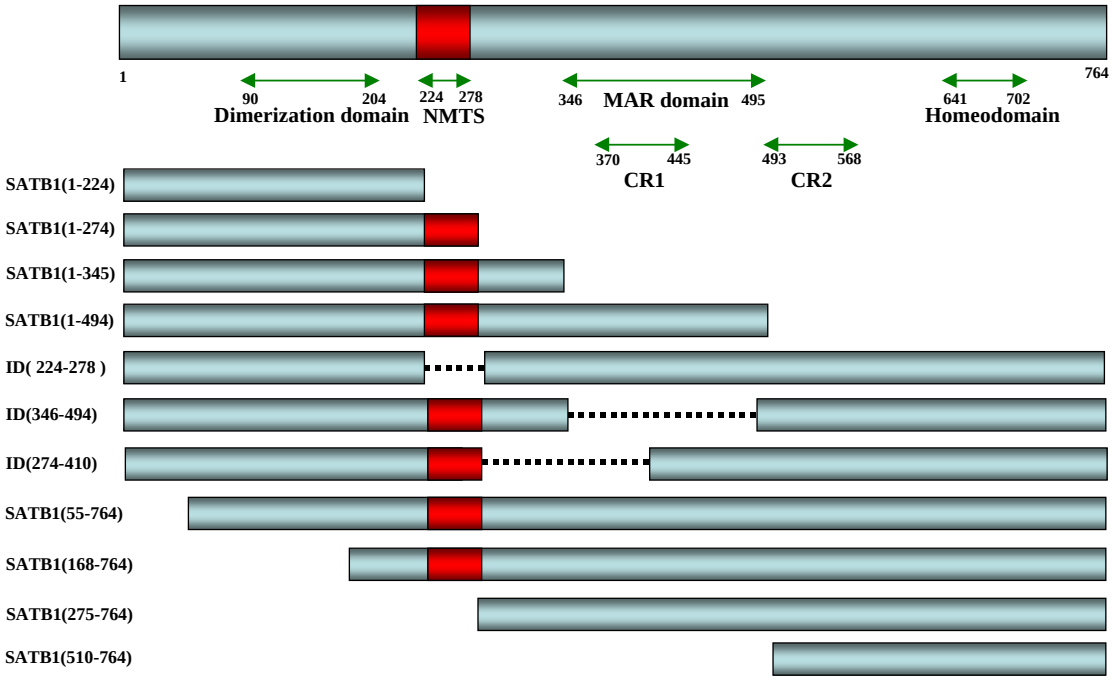


Fig. 14. Schematic diagram of SATB1 and deletion mutants.

Positions of the dimerization domain, the proposed nuclear matrix targeting sequence (NMTS), the MAR-binding domain, the Cut-like repeats (CR1 and CR2), and the homeodomain are indicated. The numbers in the N-terminal or C-terminal deletion constructs indicate the amino acids present in the construct, while the numbers in the internal deletion (ID) mutants indicate the amino acids deleted from the mutants.

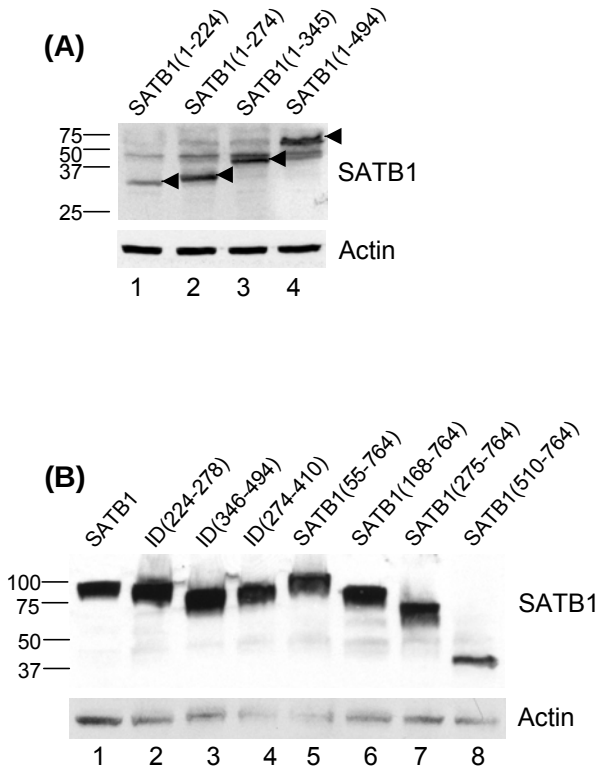


Fig. 15. Expression of wild-type and mutant SATB1 proteins.

MCF-7 human mammary cells were transfected with wild-type or mutant SATB1 expression vectors and incubated for 24 to 48 hr prior to preparation of whole cell extracts. (A) Western blotting of protein extracts with polyclonal anti-SATB1 sera specific for the N-terminus. (B) Western blotting of protein extracts with monoclonal SATB1 antibody specific for the C-terminus. Arrowheads in (A) show the expected size of SATB1 mutants. Lower panels in (A) and (B) show Western blotting with actin-specific antibody to control for protein loading.

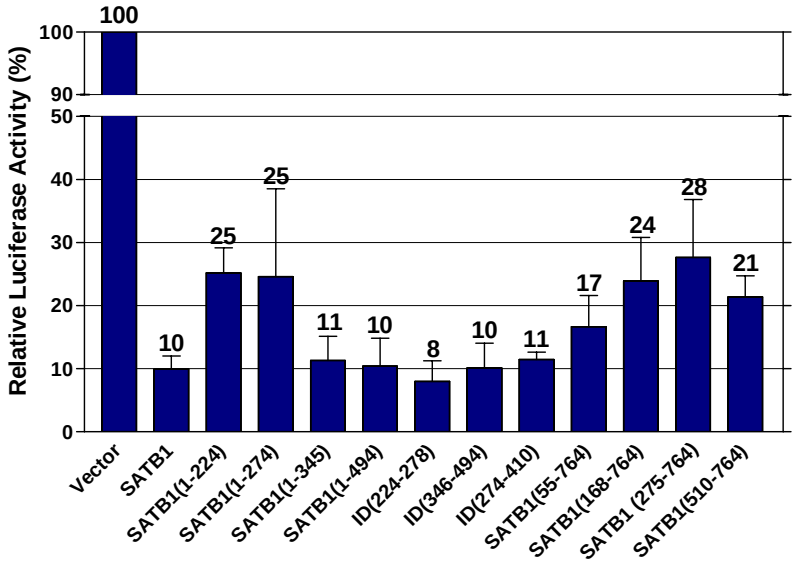


Fig. 16. DNA binding is not required for SATB1-mediated repression of the MMTV LTR from unintegrated templates.

Wild-type and mutant SATB1 expression plasmids or the empty vector were co-transfected with pMTV-LUC into MCF-7 cells in triplicate. After 48 hr, cytoplasmic extracts were prepared and a constant amount of protein was assayed for firefly luciferase activity. Results from vector-transfected cells were assigned a value of 100, and all other results are reported relative to 100.

3.1.2 SATB1 specifically represses transcription of integrated MMTV LTR-reporter plasmids and MMTV proviruses.

Integrated MMTV proviruses contain LTRs that are organized as a phased nucleosomal structure, which is absent from transiently transfected constructs containing the MMTV LTR (7,144). The response of the phased nucleosomal LTR structure to cAMP and transcription factors clearly is different from that present on

transiently introduced DNA (7,144). SATB1 has been proposed to function through HDAC recruitment and chromatin reorganization (218). Therefore, the response to SATB1 of templates containing integrated MMTV LTRs was assessed.

XC cells stably transfected with an MMTV LTR-luciferase construct (XC/MTV-LUC) were used for SATB1 overexpression experiments. To determine the effects of SATB1 on the stably integrated reporter gene, SATB1 must be expressed in a high percentage of the cells; if the percentage of expressing cells is low, the effects would be diluted by the luciferase activity of cells lacking SATB1 expression. The MigR1 retroviral expression vector system was chosen since it has successfully been used to give expression in >90% of infected cells.

The full-length SATB1 cDNA was cloned into the MigR1 vector and transiently transfected into 293T cells together with the *gag-pol* (pHIT 60) and *env* (pHIT G) expression vectors. The MigR1 vector lacking an insert was used to produce control virus. Virus particles were collected from the growth medium, concentrated by ultracentrifugation, and the virus titer was determined by FACS analysis. The MigR1 vector has an IRES-GFP cassette located downstream of the multiple cloning site so that GFP-positive cells indicate that cells have been transduced. Equal titers of virus were used for infection of XC/MTV-LUC cells. Vector-transduced and SATB1-transduced cells had comparable transduction efficiencies (93% and 94%, respectively).

As expected, addition of glucocorticoids gave a 7 to 8-fold increase in expression from the MMTV LTR in vector-transduced cells. However, surprisingly,

SATB1 suppressed MMTV LTR-luciferase activity 2-fold in the presence of DEX, but not in the absence of the hormone (Fig. 17).

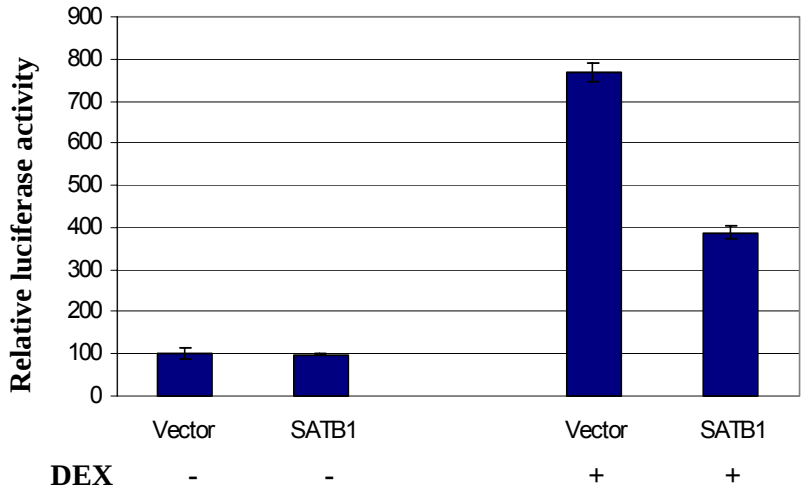


Fig. 17. SATB1 suppresses expression from the integrated MMTV LTR in the presence of glucocorticoids in XC rat cells.

XC cells stably transfected with an pMTV LTR-reporter plasmid were transduced with MigR1 or MigR1-SATB1 (MOI=2). The transduction efficiency was >90% in both cases. The luciferase values are indicated relative to a value of 100 assigned to vector-transfected cells in the absence of glucocorticoids.

To determine the dose dependence of SATB1-mediated MMTV repression, XC/MTV-LUC cells were transduced at two different MOIs. Suppression by SATB1 increased using a higher MOI (Fig. 18A), but only was observed in the presence of glucocorticoids. SATB1 suppressed luciferase expression 2.6-fold at MOI=2 and 4.4-

fold at MOI=8, and this difference was statistically significant. Western blotting confirmed that the higher MOI gave increased SATB1 expression relative to levels of actin (Fig. 18B).

A similar approach was used to determine the effect of SATB1 overexpression in XC rat cells infected by wild-type MMTV (also called XC/C3H). Rat cells lack endogenous MMTV sequences, which might complicate the analysis. XC/MMTV cells were transduced with MigR1 or MigR1-SATB1, incubated in the presence or absence of DEX for 24 hr, and then total RNA was extracted. Semi-quantitative reverse transcription (RT)-PCR using *gag*-specific primers revealed MMTV expression in the vector-transduced, but not SATB1-transduced cells, consistent with SATB1 suppression of MMTV RNA levels (Fig. 19A). However, *gag-pol* mRNA levels in vector- and SATB1-transduced cells were similar in the absence of DEX (Fig. 19B). Viral RNA expression was normalized to *gapdh* RNA levels in each reaction.

To determine the effect of SATB1 on MMTV expression more quantitatively, RNase protection assays (RPAs) were performed. MigR1 or MigR1-SATB1 particles were transduced into XC/MMTV cells to give transduction efficiencies greater than 90%. Cells were grown in the presence or absence of glucocorticoids for 24 hr prior to extraction of total RNA. RNA was hybridized simultaneously with RNA probes specific for MMTV and *gapdh* and then digested with RNase A and T1. After normalization to *gapdh* RNA levels, SATB1 suppressed MMTV expression 2.8-fold in the presence, but not the absence, of DEX (Fig. 20).

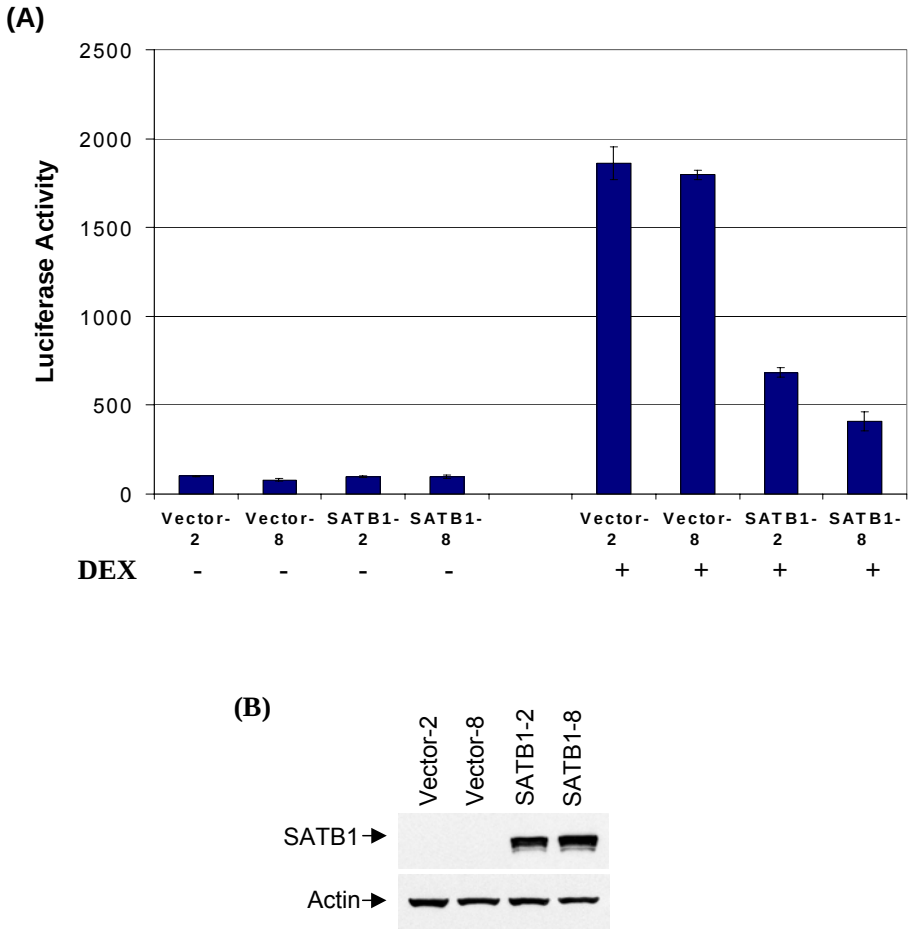


Fig. 18. SATB1 suppresses stably transfected MMTV LTR-luciferase expression in a dose-dependent manner.

XC/MTV-LUC cells were transduced with MigR1 or MigR1-SATB1 virus at an MOI=2 or MOI=8 as indicated. (A) SATB1-mediated suppression of MMTV LTR expression is observed only in the presence of glucocorticoids. The luciferase values were reported relative to a value of 100 for vector-transduced cells at MOI=2. (B) Western blot analysis of SATB1 levels in transduced XC/MTV-LUC cells.

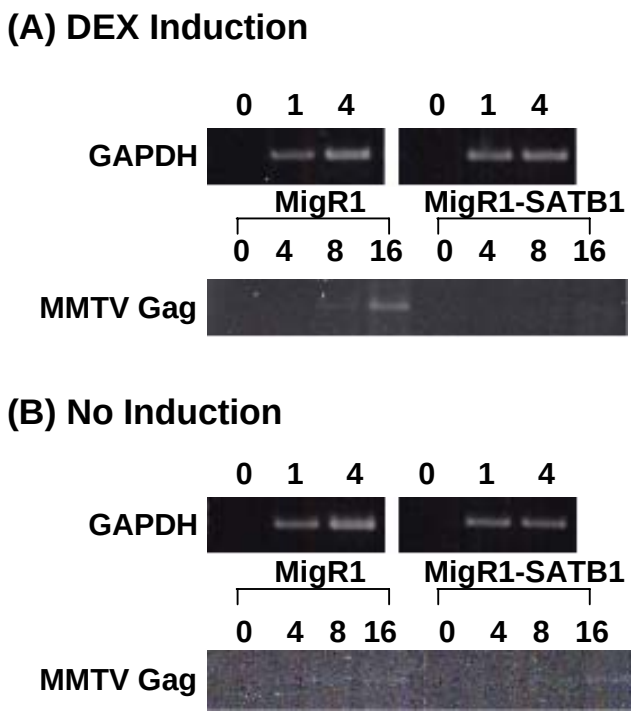


Fig. 19. SATB1 suppresses expression of integrated MMTV proviruses in XC cells in the presence of DEX.

XC/MMTV cells were transduced with MigR1 or MigR1-SATB1. Total RNA was extracted and subjected to semi-quantitative RT-PCR with *gag*-specific primers and different amounts of cDNA (indicated in microliters above each lane). RT-PCR with *gapdh*-specific primers was used to normalize for the amount of RNA in each reaction. (A) MMTV *gag-pol* mRNA expression in the presence of glucocorticoids. (B) MMTV *gag-pol* mRNA expression in the absence of glucocortioids.

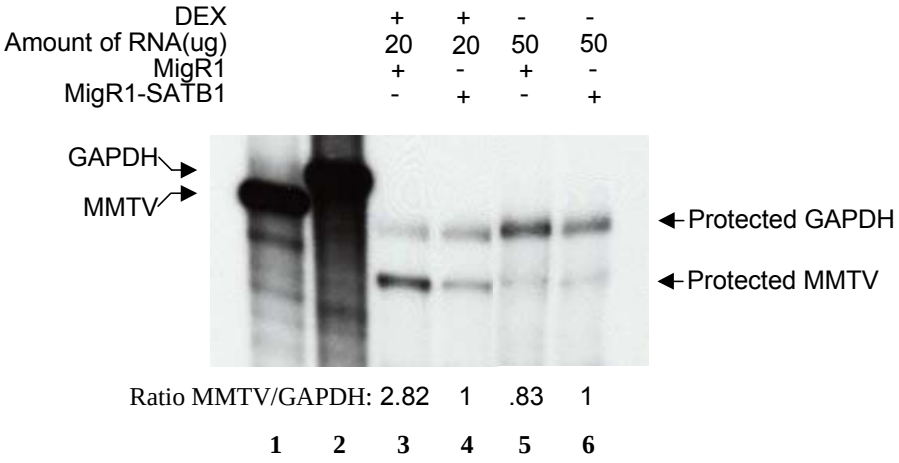


Fig. 20. RPAs indicate that SATB1 represses MMTV RNA expression in XC cells only in the presence of glucocorticoids.

XC/MMTV cells were super-infected with MigR1 or MigR1-SATB1 and then grown in the presence or absence of 10^{-6} M DEX for 24 hr. Total RNA was extracted and used for an RPA with an MMTV LTR-specific probe. The numbers below the figure indicate the ratio of MMTV RNA in MigR1-transduced cells relative to MigR1-SATB1-transduced cells after normalization for *gapdh* RNA levels. Ratios were calculated independently for cells treated with or without DEX.

The observation that SATB1 affects MMTV RNA levels in rat XC cells only in the presence of glucocorticoids is not consistent with previous studies of MMTV expression (25,124). To determine if the results in XC cells are consistent with SATB1 effects in other cells, a mouse T-cell line (BW5147) and mouse mammary epithelial cell line (HC11) were examined. Both BW5147 and HC11 were stably transfected with pMTV-LUC following hygromycin selection to give BW5147/MTV-LUC and HC11/MTV-

LUC. Cells were transduced with either MigR1 or MigR1-SATB1 virus to give greater than 90% transduction efficiencies (data not shown) prior to growth in the presence and absence of DEX. Western blotting confirmed SATB1 overexpression (Fig. 21C). Unlike the XC cells, SATB1 suppressed pMTV LTR-luciferase expression in both the mouse T-cell and mammary cell lines, regardless of the presence of glucocorticoids (Figs. 21A and B). However, HC11 cells showed greater suppression of luciferase expression than that observed in BW5147 cells, probably because HC11/SATB1 cells had higher relative levels of SATB1 expression.

These results suggested that the level of SATB1 in transduced cells might affect the ability of SATB1 to repress MMTV transcription in XC cells in the absence of glucocorticoids. To test this idea, XC/MTV-LUC cells were transduced with MigR1 and MigR1-SATB1 at a higher MOI. Again, SATB1 repression of MMTV LTR-luciferase expression was observed only in the presence of DEX (data not shown). These data suggest that XC cells have a specific defect in their ability to respond to SATB1 in the absence of glucocorticoids.

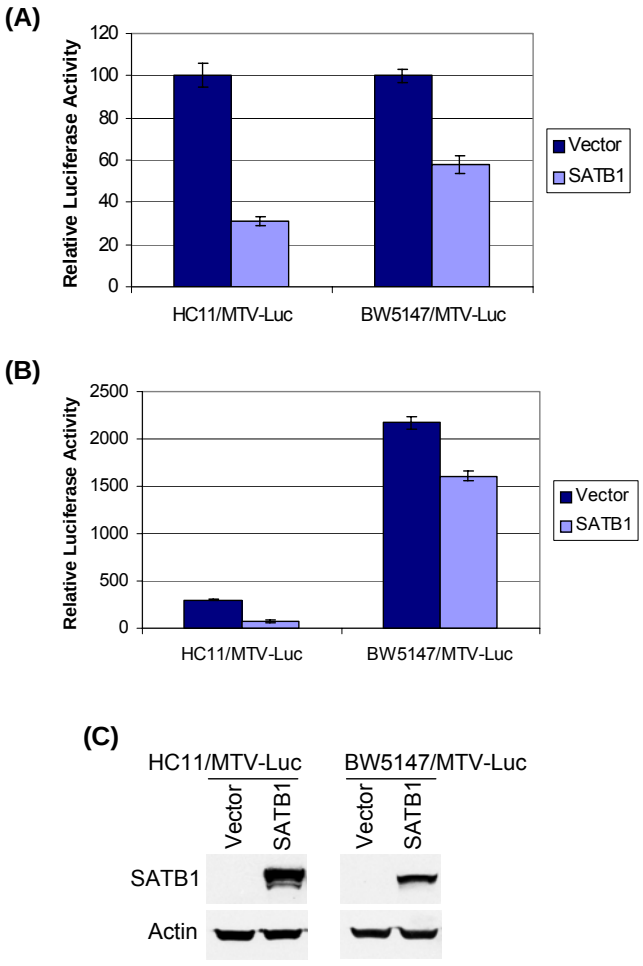


Fig. 21. SATB1 represses MMTV LTR-luciferase expression in mouse T-cell and mammary epithelial cell lines in the presence and absence of DEX.

HC11/MTV-LUC and BW5147/MTV-LUC cells were transduced with MigR1 and MigR1-SATB1 viruses at MOI=15 and MOI=30, respectively, prior to growth for 24 hr in the absence (A) or presence (B) of 10^{-6} M DEX. The data represent the means ± standard deviations of three independent luciferase determinations. The luciferase value of MigR1-transduced cells was set to 100, and the values of SATB1-transduced cells are expressed relative to 100. The luciferase activities of cells transduced with vector and SATB1 infectious particle in each set are statistically different by the Student's *t* test ($P < 0.01$). (A) SATB1 suppresses MMTV expression in the absence of DEX in HC11 and BW5147 cells. (B) SATB1 suppresses MMTV expression in the presence of DEX in HC11 and BW5147 cells. (C) Western blot analysis of SATB1 expression in transduced HC11 and BW5147 cells.

3.1.3. The SATB1 DNA-binding domain is required for SATB1-mediated repression of transcription from integrated MMTV LTRs.

To determine the specificity of SATB1 suppression on the integrated MMTV LTR, vectors expressing wild-type SATB1 or a DNA-binding domain mutant (ΔDBD) [ID(346-494)] or the empty MigR1 vector were transduced into XC cells infected by C3H MMTV (XC/MMTV). Infected cells were treated for 24 hr in the presence or absence of glucocorticoids, and then extracted for total RNA. As expected, the wild-type SATB1 gave ca. 2-fold suppression of reporter gene expression relative to that from MigR1-transduced cells (Fig. 22). Deletion of the major SATB1 DBD relieved suppression of SATB1-mediated repression of transcription in the presence, but not the absence, of glucocorticoids. This experiment was repeated in HC11 mammary cells containing an integrated MMTV LTR-reporter gene (Fig. 23). Similar to results with XC cells, SATB1 suppressed expression from the LTR in the presence of DEX, and this suppression was relieved by deletion of the major DNA-binding domain. However, unlike experiments in XC cells, SATB1-mediated repression was observed in the presence or absence of DEX. These data indicate that DNA binding is required for SATB1 function on integrated MMTV LTR templates.

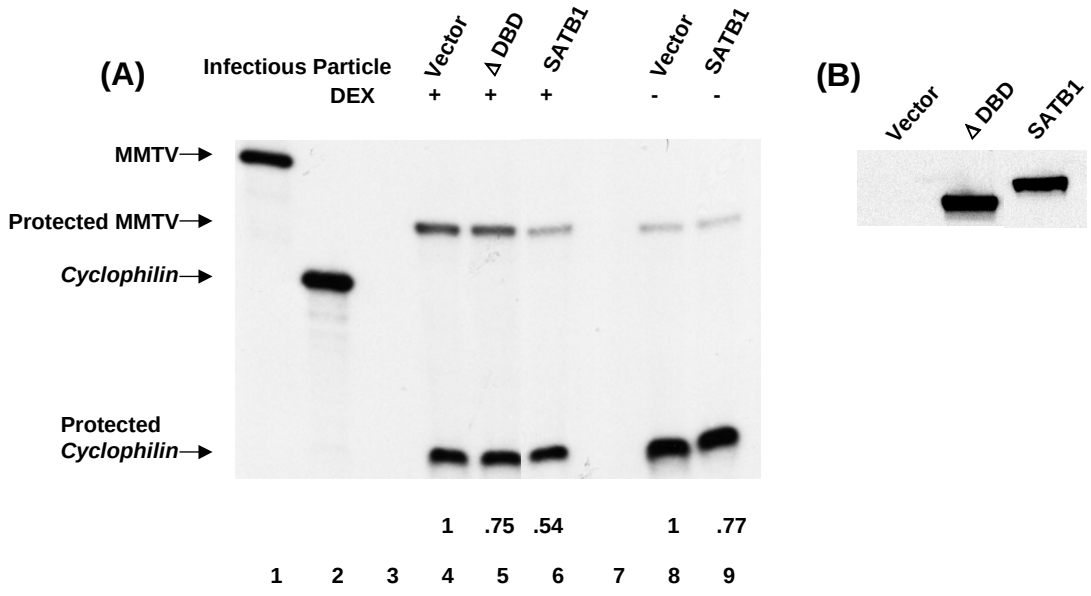


Fig. 22. DNA binding is required for SATB1-mediated repression of expression from integrated MMTV proviruses in XC cells.

XC/MMTV cells were transduced with MigR1-SATB1, MigR1-ΔDBD [ID(346-494)], or MigR1 virus. After transduction, half of the cells were treated for 24 hr with 10^{-6} M DEX. Total RNA was extracted for RNase protection assays using an MMTV LTR probe. A portion of the cells also was used for protein extracts. (A) RNase protection assays of RNA extracted from MMTV-infected cells transduced by viruses expressing wild-type and mutant SATB1 proteins. RNA was obtained from cells grown in the presence (lanes 4 to 6) or absence (lanes 8 and 9) of DEX; the amounts used for hybridization were 15 μg RNA from hormone-treated cells and 70 μg RNA from untreated cells. The positions of full-length and protected MMTV and cyclophilin (control) probes are shown by arrows. The ratio of MMTV to cyclophilin RNA expression was determined by phosphorimaging. (B) Western blotting of wild-type and ΔDBD SATB1 proteins in transduced cells.

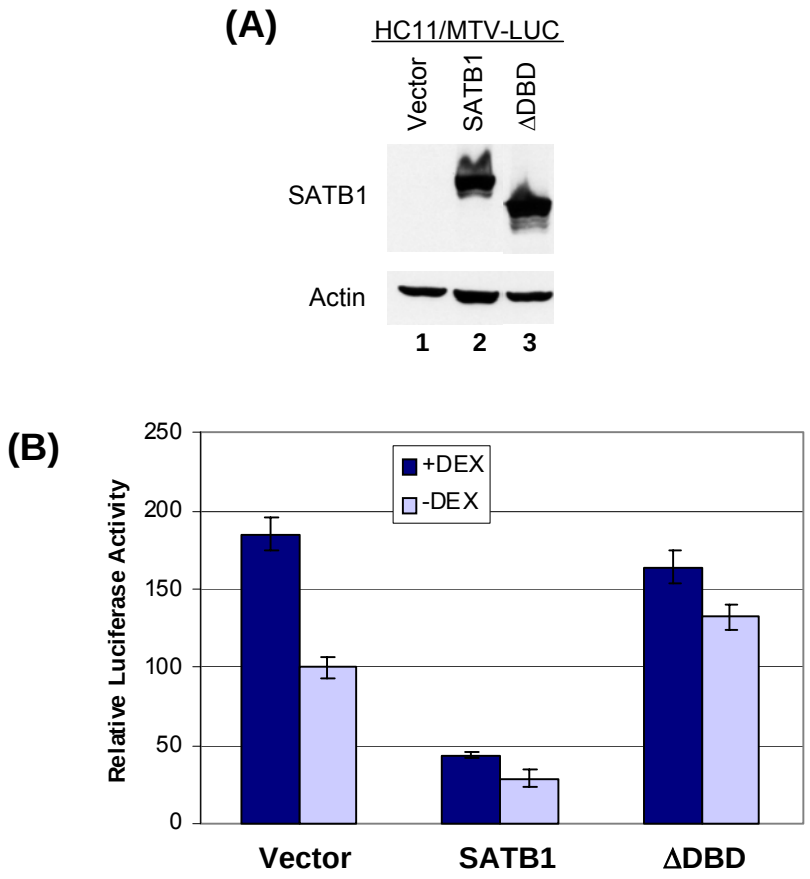


Fig. 23. DNA binding is required for SATB1-mediated repression of MMTV LTR-reporter gene expression in HC11 cells.

HC11/MTV-LUC cells were transduced with MigR1-SATB1, MigR1-ΔDBD, or MigR1 virus. After transduction, the cells were expanded for 7 days and treated for 1 hr with 10^{-6} M DEX. (A) Western blotting of wild-type and ΔDBD [ID(346-494)] SATB1 proteins in transduced cells. (B) The data represent the means ± standard deviations of three independent luciferase determinations. The luciferase value of MigR1-transduced cells without hormone induction was set to 100, and the values of SATB1-transduced cells are expressed relative to 100. The luciferase activities of cells transduced with vector, SATB1, and ΔDBD infectious particle in each set of in the presence and absence of DEX are statistically different by the Student's *t* test ($P<0.01$).

3.1.4. Knock-down of endogenous SATB1 levels in Jurkat T cells elevates MMTV expression.

The Jurkat human T-cell line has high endogenous levels of SATB1 (Fig. 6). Therefore, the small-interfering (si) RNA technique was used to determine if reduction of SATB1 levels in these cells would elevate MMTV expression. Jurkat cells were stably transfected with the MMTV-LUC expression plasmid and then incubated with 600 nM SATB1-specific siRNAs or a non-targeting siRNA in the presence of glucocorticoids. After 60 hr, cells were analyzed by Western blotting and for luciferase activity (Fig. 24). Western blotting revealed approximately 3-fold suppression of SATB1 levels in cells treated with the targeting siRNA versus cells treated with the non-targeting siRNA (Fig. 24A). Moreover, treatment with the SATB1-specific siRNA increased MMTV LTR-luciferase levels ca. 1.5-fold relative to that from cells treated with the non-targeting siRNA (Fig. 24B). These data are consistent with previous SATB1 overexpression experiments indicating that SATB1 represses transcription from the integrated MMTV LTR promoter.

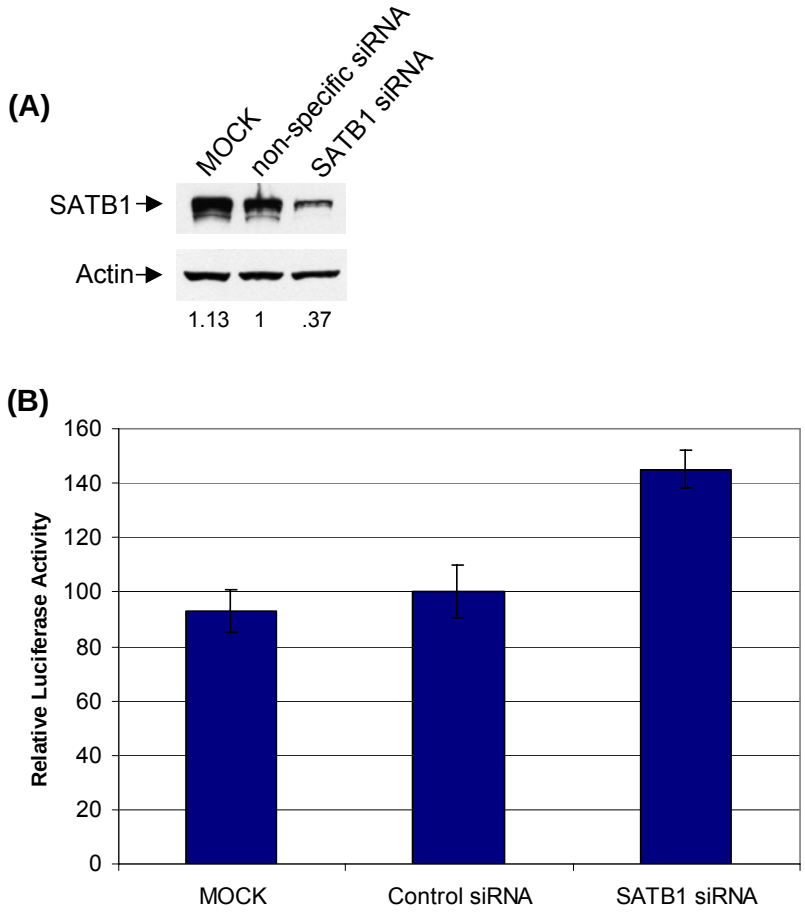


Fig. 24. Knock-down of endogenous SATB1 expression elevates MMTV expression in Jurkat/MTV-LUC cells.

Jurkat/MTV-LUC cells were electroporated with 600 nM SATB1-specific siRNA or a non-targeting siRNA. Mock treatment consisted of electroporation in the absence of siRNA. Cells were incubated for 60 hr prior to preparation of protein extracts. (A) Western blotting with SATB1-specific monoclonal antibody (upper panel) shows decreased SATB1 levels relative to actin levels (lower panel) after specific siRNA treatment. (B) SATB1-specific siRNA treatment leads to increased luciferase expression from the MMTV LTR. Luciferase levels in cells treated with the non-targeting siRNA were assigned to a value of 100, and values determined for the same amount of protein in other samples are given relative to 100. Values are means of triplicate determinations ± standard deviation. The luciferase activities of cells treated with control and SATB1-specific siRNAs are statistically different by the Student's *t* test ($P<0.01$).

3.2 THE DOMAIN STRUCTURE OF SATB1

The previous section used overexpression and knock-down experiments to show that SATB1 functions to suppress transcription from integrated templates containing the MMTV LTR. In this section, the previously described DNA-binding and dimerization domains of SATB1 were verified, and several new protein localization domains were defined.

3.2.1. Domain requirements for SATB1 binding to the MMTV NRE.

Previous data indicated that the major domain required for SATB1-binding to DNA encompassed the MAR-binding domain (52). The dimerization domain from amino acids 90 to 204 also was required for DNA binding (68). To test the ability of SATB1 deletion mutants to bind DNA, nuclear extracts were prepared from cells that had been transfected with wild-type and mutant SATB1 plasmids. Nuclear extracts were then used in EMSAs with the promoter-proximal NRE4 probe, which has four repeats of the strong SATB1 binding site located between -287 and -265 in the MMTV LTR (124). The related transcription factor, CDP, appears to have a weaker affinity for this probe (124).

As anticipated, wild-type SATB1 showed binding to the NRE probe (Fig. 25, lane 3), and at least two DNA-protein complexes were observed. The C-terminal truncation mutants (1-224, 1-274, and 1-345), which lack the MAR domain, CR1, CR2, and the homeodomain, did not bind DNA (lanes 4-6). Internal deletions ID(346-494) and ID(274-410) missing the MAR domain also had no detectable DNA binding. The N-terminal deletion mutants, SATB1(168-764), (274-764), and (510-764) did not bind

NRE4, consistent with previous data that the dimerization domain is required for SATB1 binding to DNA (68). Notably, SATB1(55-764) showed reduced binding to the NRE, particularly the lower mobility complex, suggesting that this mutation may affect the efficiency of protein multimerization. An internal deletion mutant, ID(224-278), had approximately wild-type DNA-binding activity (compare lanes 3 and 8), although the intensity of the lower mobility complex was increased compared to the wild-type protein. SATB1(1-494), which contains both the MAR-binding and dimerization domains, showed limited binding to the NRE4 probe.

To determine the identity of complexes formed with the MMTV NRE, we used antibody ablation experiments (Fig. 26). Anti-SATB1, but not preimmune serum, abolished NRE binding of all SATB1-specific complexes observed. The nuclear extract of Jurkat cells, which express high levels endogenous SATB1, was used for a positive control. The results showed that endogenous SATB1 and SATB1 in MCF-7 cells migrated similarly (Fig. 26, lanes 12-15), and the observed bands were abolished by SATB1-specific antibody. Similar results were observed when the rat fibroblast cell line, XC, was used for transfection and gel shift assays (data not shown).

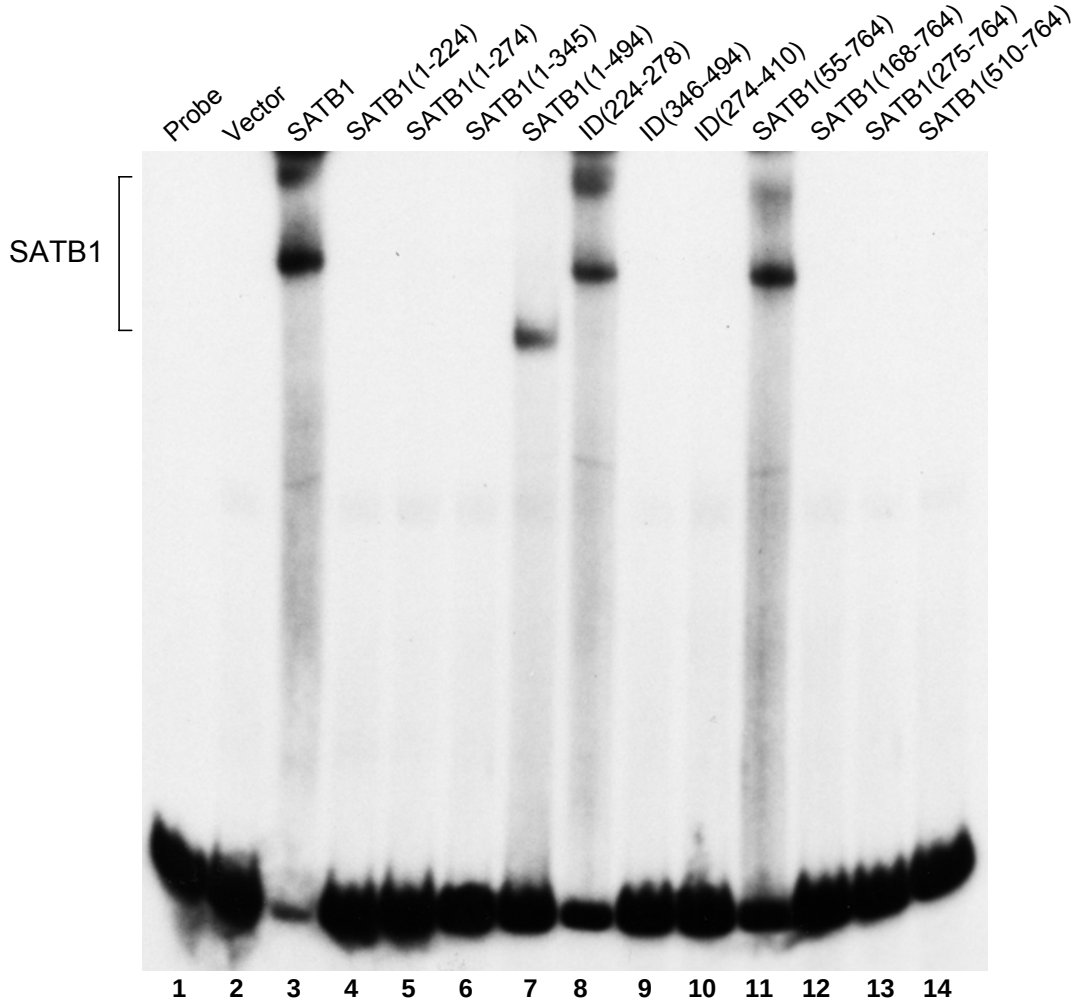


Fig. 25. Dimerization and MAR-binding domains are required for SATB1 binding to the MMTV NRE.

EMSAs using extracts from cells expressing wild-type and mutant SATB1 proteins. Nuclear extracts were prepared from MCF-7 human mammary cells transfected with SATB1 expression plasmids. Extracts then were used in gel shift assays with NRE4 probe, a four-repeat concatamer of strong SATB1-binding sites in the MMTV NRE. Lane 1 contains the labeled probe in the absence of added nuclear extract, whereas lane 2 shows probe binding to nuclear extracts transfected with the empty vector. Gel shifts with nuclear extracts obtained from transfections with wild-type SATB1 (lane 3) or various mutants (lanes 4 to 14) are shown. A bracket shows the distribution of various SATB1-specific complexes.

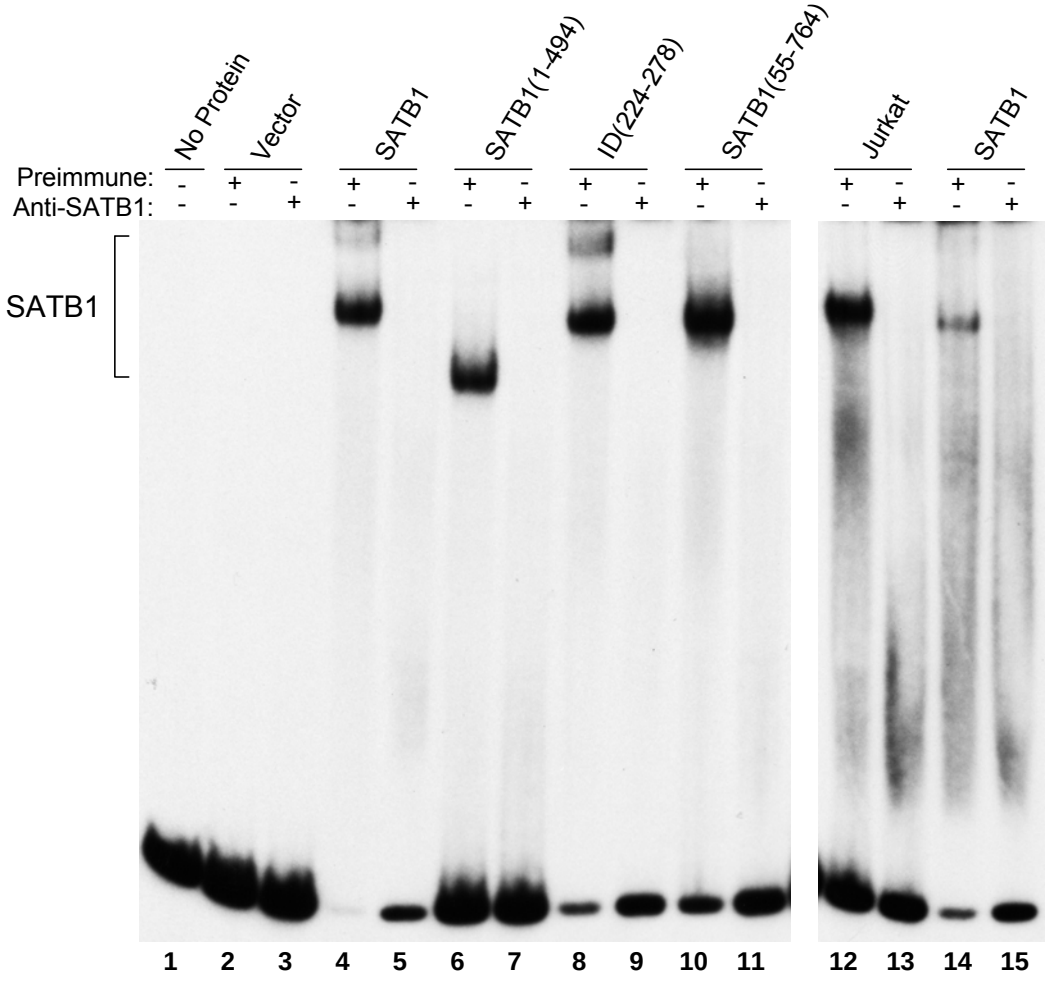


Fig. 26. Antibody ablation assays reveal the identity of the retarded bands.

Even-numbered lanes indicate assays performed in the presence of preimmune sera and odd-numbered lanes (except lane 1) indicate assays performed in the presence of SATB1-specific rabbit polyclonal antibody. Gel shifts with nuclear extract from human Jurkat T cells (lanes 12 and 13) were used as a positive control. Lanes 2-11, 14 and 15 contain nuclear extract from transfected MCF-7 cells. Gel shifts shown in lanes 4 and 5 and lanes 14 and 15 were performed using nuclear extracts from different transfections with wild-type SATB1.

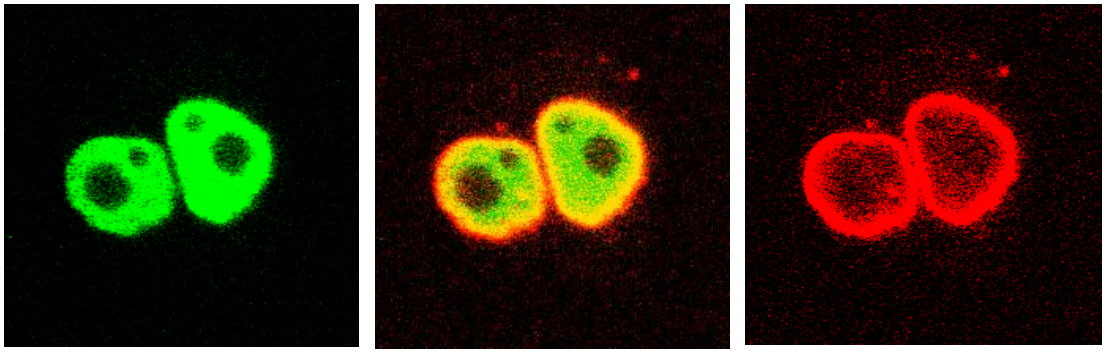
3.2.2. Subnuclear localization of SATB1 and deletion mutants.

SATB1 previously has been shown to be a nuclear protein with MAR-binding activity (50). To determine the subnuclear distribution of SATB1, confocal microscopy was performed in conjunction with staining for lamin B, a nuclear matrix protein (88). A GFP-SATB1 fusion construct was prepared and transfected into MCF-7 cells grown on glass cover slips. Cells were fixed, permeabilized, and stained with lamin B-specific antibody. The primary antibody then was detected by incubation with a rhodamine-conjugated secondary antibody.

GFP-SATB1 was diffusely localized inside the nucleus, with the exception of the nucleolus, whereas lamin B was largely concentrated at the inner surface of the nuclear membrane. A merged image showed that SATB1 overlaps the inner half of the ring structure formed by lamin B (Fig. 27). Since lamin B is a major component of the nuclear matrix (88), these results suggested that a portion of wild-type SATB1 was associated with the nuclear matrix.

To test whether the nuclear matrix association of SATB1 is determined by a particular protein domain, wild-type SATB1 and deletion mutants were analyzed by biochemical fractionation. To validate this method, MCF-7 cells were fractionated into soluble, chromatin, and nuclear matrix fractions based on the procedure of He *et al*. (50,111). The soluble fraction (SF) contains cytosolic and soluble nuclear proteins, the chromatin (CH) fraction contains proteins that tightly associate with chromatin, and the nuclear matrix (NM) fraction contains nuclear matrix and membrane-bound proteins. To verify the efficiency of the method, fractions were subjected to Western blotting and incubation with antibodies specific for lamin B, histone H1, and cytochrome C (a

mitochondrial protein) (152) (Fig. 28). As expected, lamin B, histone H1, and cytochrome C were detected only in the nuclear matrix, chromatin, and soluble fractions, respectively. These results verified the quality of the fractions obtained.



GFP-SATB1 Merge LaminB

Fig. 27. SATB1 is a nuclear protein that partially colocalizes with lamin B.
MCF-7 cells grown on glass cover slips were transfected with GFP-SATB1 expression plasmid and incubated for 24 hr. Cells then were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were stained with lamin B-specific antibody, washed, and incubated with secondary antibody conjugated with rhodamine. The cover slips were observed under a Leica TCS4D confocal microscope.

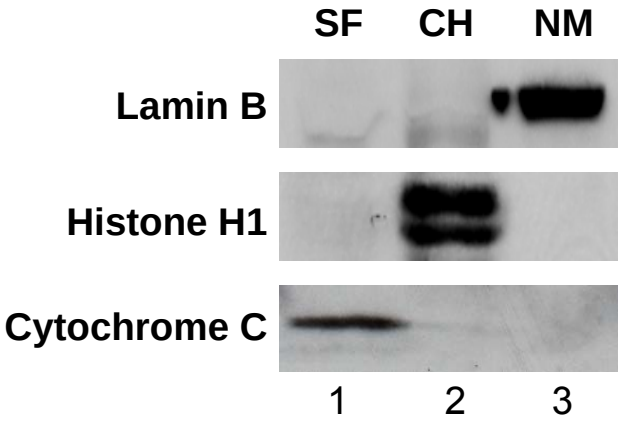


Fig. 28. Subcellular fractionation of various endogenous cellular proteins.
MCF-7 cells were incubated with Triton X-100 in CSK buffer to extract soluble cytoplasmic and nuclear proteins (SF). The insoluble fraction was treated with DNase I to remove DNA, and soluble chromosome-bound proteins were obtained (CH). The remaining insoluble proteins were washed extensively with 2 M NaCl, and then 8M urea was added to obtain the nuclear matrix fraction (NM). Fractions were resolved on SDS-containing 10% polyacrylamide gels and subjected to Western blotting. Blots were incubated with antibodies specific for lamin B (upper panel), histone H1 (middle panel), or cytochrome C (lower panel).

To determine the subcellular localization of SATB1 deletion mutants, MCF-7 cells were transfected with wild-type and mutant constructs and subjected to biochemical fractionation (Fig. 29). Each fraction was subjected to Western blotting with SATB1-specific antibody. Wild-type SATB1 localized in relatively equal proportions to the soluble, chromatin and nuclear matrix fractions. However, SATB1(1-494), missing the entire C-terminus of the protein, redistributed so that none of the protein localized in the soluble fraction. Further C-terminal deletion to amino acid 274 gave a protein that remained associated with the nuclear matrix, although the amount

in the matrix appeared to be diminished. However, the mutant SATB1(1-224) appeared to be localized entirely to the soluble fraction. These results implicated amino acids 224 to 278 of SATB1 as a nuclear matrix targeting sequence (NMTS).

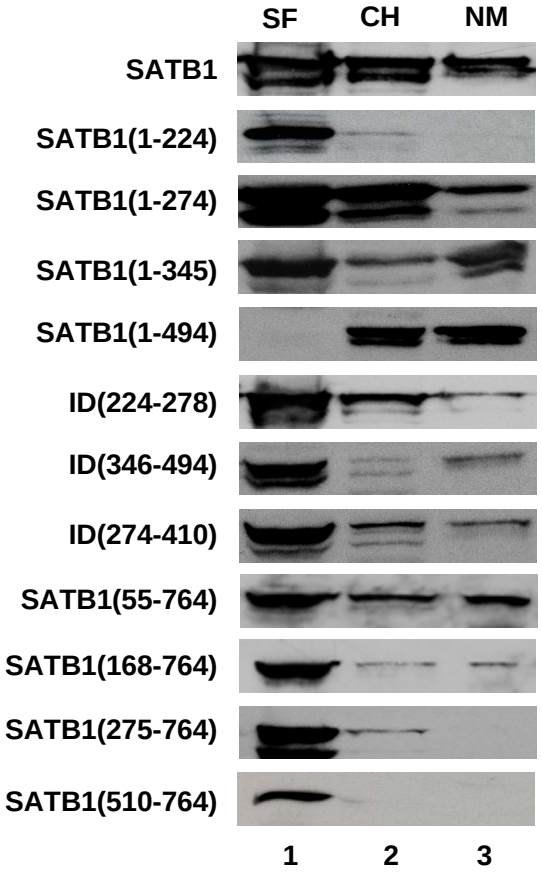


Fig. 29. Subcellular localization of wild-type and mutant SATB1 proteins.

SATB1 wild-type and mutant proteins were transfected into MCF-7 cells, incubated for 24 to 48 hr, and fractionated. Equal amounts of each fraction were separated on denaturing polyacrylamide gels and subjected to Western blotting. Extracts from the internal and N-terminal deletion mutants were incubated with monoclonal SATB1-specific antibody, whereas extracts from cells transfected with the C-terminal deletion mutants were incubated with polyclonal sera specific for the SATB1 N-terminus.

Because the biochemical fractionation of He et al. (81) does not distinguish between soluble cytoplasmic and nuclear proteins, immunofluorescence microscopy was used to verify that the loss of nuclear matrix association by the mutant SATB1(1-224) protein was not due to failure to localize to the nucleus. MCF-7 cells were transfected with the mutant construct and cultured for approximately 48 hr. Cells then were fixed, incubated with polyclonal anti-SATB1 sera, washed, and incubated with specific secondary antibodies and DAPI. Results of these experiments indicated that the mutant SATB1 was co-localized with the DAPI-stained nuclei (Fig. 30). These data indicate that the N-terminal 224 amino acids of SATB1 contain a nuclear localization signal (NLS), but not an NMTS.

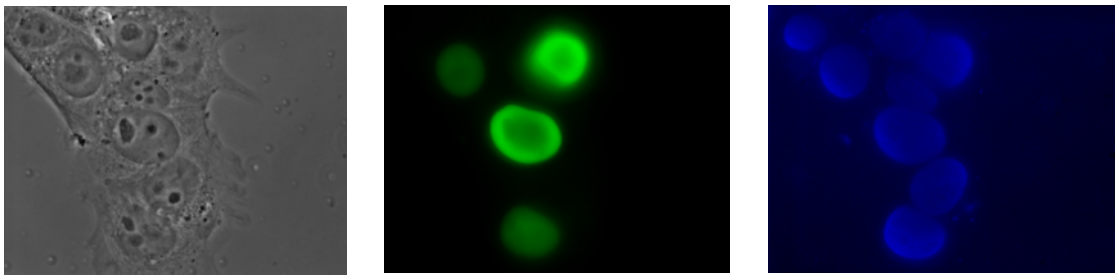


Fig. 30. The SATB1 mutant (1-224) is localized to the nucleus.
MCF-7 cells were grown on glass discs, transfected with SATB1(1-274) expression vector and incubated for 48 hr. The cells were fixed with 4% paraformaldehyde in PBS, permeabilized, and stained with SATB1-specific polyclonal antibody. The cells then were incubated with FITC-labeled anti-rabbit IgG. DAPI staining was used to localize the nuclei.

A series of N-terminal deletion mutants were tested to confirm the localization of an NMTS to the SATB1 region between amino acids 224 to 278 (Fig. 29). The 54 N-terminal amino acids, which have been reported to be cytotoxic in yeast (68), were deleted. However, this mutant (55-764), localized similar to that of wild-type SATB1. Further deletions to amino acids 168 or 275 greatly reduced chromatin and nuclear matrix association, consistent with an NLS in this region. The mutant SATB1(275-764) appeared to have no nuclear matrix association, consistent with an NMTS localized between amino acids 224 to 278. Furthermore, fluorescent microscopy showed that nuclear localization of SATB1(168-764) was reduced compared to wild type SATB1 although the deletion did not completely abolish its nuclear localization (data not shown).

Internal deletion (ID) mutants also were constructed to aid localization of SATB1 regions that determine nuclear matrix association. ID(224-278), which deletes the putative NMTS, significantly reduced, but did not abolish, the nuclear matrix localization of SATB1 (Fig. 29). Similarly, examination of the mutants ID(274-410) and ID(346-494) suggested that deletion of the major DNA-binding domain also reduced nuclear matrix association. These data suggest that SATB1 may contain multiple nuclear matrix localization sequences. Because the major DNA-binding domain is required independently for SATB1-mediated transcriptional repression, the NMTS between amino acids 346 and 494 was not further characterized. Additional experiments focused on the SATB1 NMTS located between 224 to 278.

3.2.3. A SATB1 NMTS is localized between amino acids 224 and 278.

To specifically determine whether the SATB1 NMTS targets non-nuclear matrix proteins to the nuclear matrix, GFP and LexA were independently fused to SATB1 sequences between amino acids 224 and 278 (GFP-NMTS and LexA-NMTS). The fused and unfused constructs were transfected into MCF-7 cells, and protein localization was analyzed by biochemical fractionation. GFP, which lacks an NLS (77), localized primarily to the soluble fraction. However, the fusion protein GFP-NMTS was distributed to the soluble, chromatin, and nuclear matrix fractions similar to wild-type SATB1 (Fig. 29 and 31). On the other hand, LexA, which has an NLS (186), localized to both the soluble and chromatin fractions. The LexA-NMTS fusion again showed relocalization of a fraction of the protein to the nuclear matrix.

To quantitate the effect of the NMTS on nuclear matrix association, Western blotting of fractions obtained from cells transfected with plasmids expressing GFP or various GFP-fusion proteins was performed (Fig. 32). The amount of these proteins in different fractions was determined using the Odyssey imaging system and compared to that of a known nuclear matrix protein, lamin B. At least 90% of lamin B was detected in the nuclear matrix in multiple sample preparations, whereas only 3% of GFP was in this fraction. However, 42% of GFP-NMTS containing SATB1 amino acids from 224 to 278 associated with the nuclear matrix, a 14-fold increase. GFP-SATB1(1-55) containing the first 55 amino acids of SATB1 showed no increased association with the nuclear matrix compared to GFP alone, whereas GFP-SATB1(1-224) showed a 4-fold increase in association with the nuclear matrix compared to GFP. The latter observation may result from increased GFP localization to the nucleus due to the NLS

or to weak NMTS activity of these sequences or both. Nevertheless, these results confirm localization of a SATB1 NMTS to a region between amino acids 224 to 278.

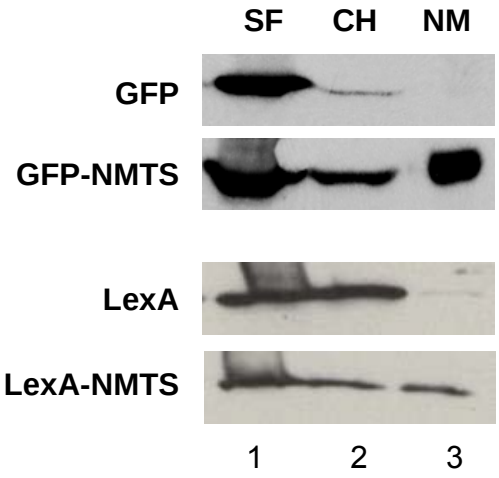


Fig. 31. A SATB1 NMTS targets heterologous proteins to the nuclear matrix.
The GFP, LexA, GFP-NMTS, and LexA-GFP expression constructs were transfected into MCF-7 cells. After 24 to 48 hr, cells were fractionated into soluble (SF), chromatin (CH), and nuclear matrix (NM) fractions. Fractions were separated on denaturing polyacrylamide gels and subjected to Western blotting. Blots were incubated with antibodies specific for LexA or GFP.

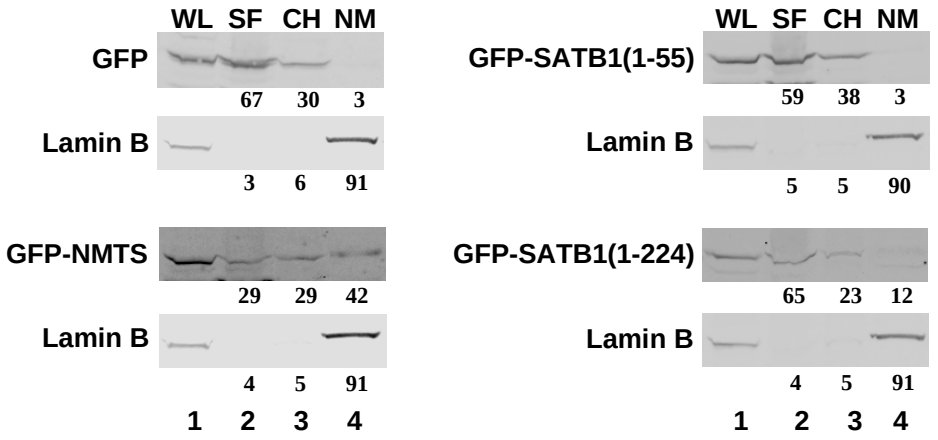


Fig. 32. Quantitation of nuclear matrix association of GFP-SATB1 fusion proteins. Plasmids encoding GFP or GFP-SATB1 fusion proteins were transfected into MCF-7 cells, incubated for 24 to 48 hr, and fractionated. Fractions were analyzed by Western blotting and incubation with GFP or lamin B-specific antibodies. Alexa Fluor 680-conjugated rabbit Ig-specific or IRDye800-conjugated goat Ig-specific antibodies were used to detect bands. Band intensities were measured using the Odyssey imaging system. The total amount of fluorescence in the soluble, chromatin, and nuclear matrix fractions was set to 100. The small numbers below each figure indicate the percentage of fluorescence of either GFP or lamin B present in each fraction. The whole cell lysate (WL) represents the amount of protein in one-quarter of the unfractionated transfected cells after sonication.

Confocal microscopy was used to confirm the biochemical fractionation experiment. MCF-7 cells grown on glass discs were transfected with LexA- or LexA-NMTS expression constructs. Transfected cells then were subjected to nuclear matrix preparation on the discs and then incubated with LexA-specific antibody. After washing, cells were then incubated with FITC-conjugated secondary antibody. The

number of cells transfected with LexA or LexA-NMTS was comparable. However, only LexA-NMTS was observed after extraction, consistent with the ability of the NMTS to direct LexA to the nuclear matrix (Fig. 33). Together with the biochemical fractionation, these data indicate that the SATB1 amino acids between 224 and 278 act as a nuclear matrix targeting sequence.

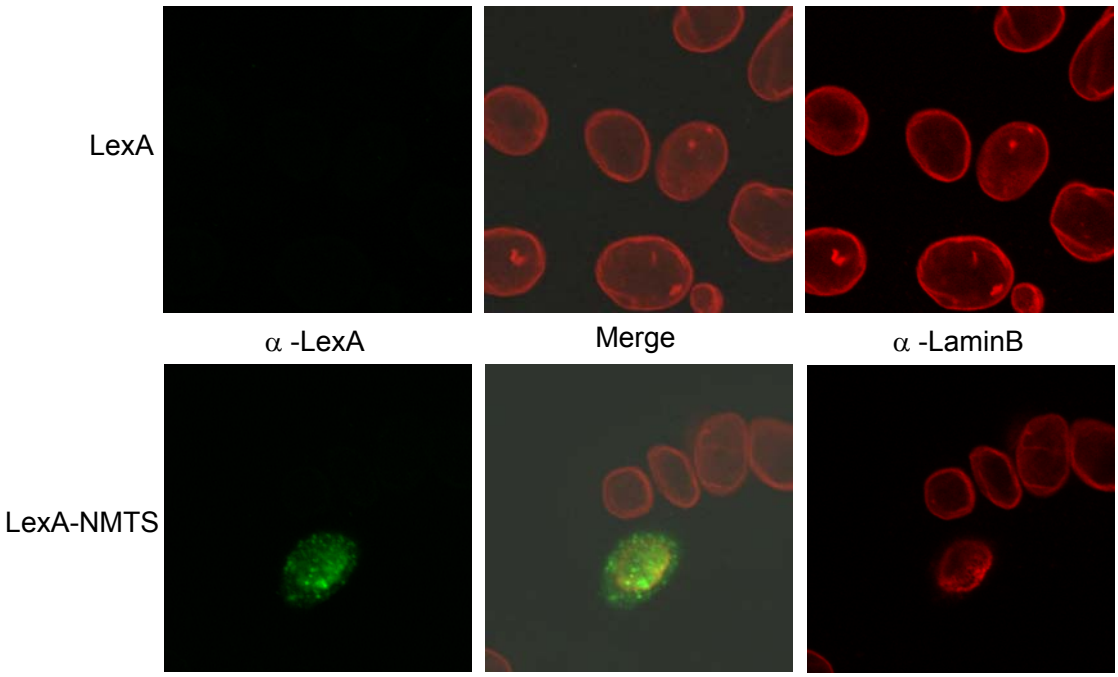


Fig. 33. The SATB1 NMTS promotes retention of LexA in the nuclear matrix.
MCF-7 cells grown on glass discs were transfected with LexA or LexA-NMTS expression vectors. Cells were incubated for 24 hr and nuclear matrix was prepared *in situ*. Subsequently, these preparations were incubated with LexA- and lamin B-specific antibodies prior to staining with FITC-conjugated mouse-specific and rhodamine-conjugated goat-specific secondary antibodies. Confocal microscopy was used to detect independent or merged images of the two fluorescent antibodies.

One explanation for the observed results is that the defined NMTS of SATB1 contains an NLS, leading to additional association of GFP-NMTS with the nuclear matrix. To eliminate the possibility, localization of a SATB1 NLS was confirmed by fluorescent microscopy of cells expressing wild-type and mutant SATB1 proteins fused to GFP (Fig. 34). GFP alone is localized throughout the cell, whereas GFP-SATB1 was strictly nuclear with a slightly punctate appearance. Linkage of the first 55 amino acids of SATB1 to GFP resulted in slightly more nuclear localization compared to GFP alone, but fusion to the N-terminal 224 amino acids had an exclusively nuclear punctate appearance as previously described (77). GFP fusion to SATB1 amino acids 224-278 (GFP-NMTS) showed a similar localization as GFP alone, suggesting that this region lacks an NLS. Since no consensus NLS is localized in the first 224 amino acids of SATB1, these results are consistent with multiple weak NLSs in this region.

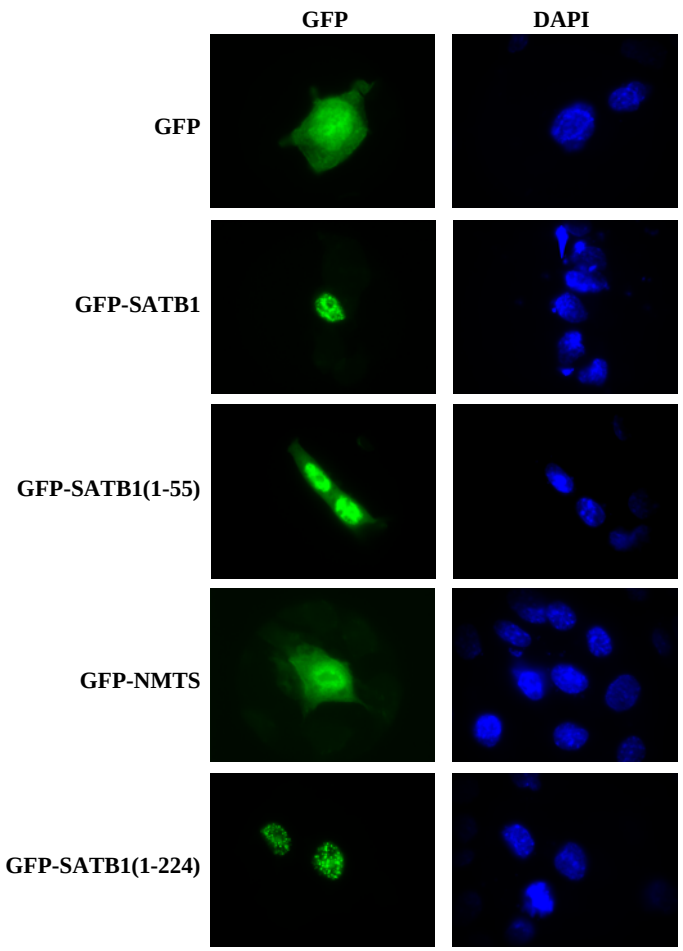


Fig. 34. Intracellular localization of wild-type and mutant SATB1 fusion proteins by fluorescence microscopy.

HC11 mouse mammary cells were transiently transfected with plasmids encoding GFP or GFP fusions to wild-type or mutant SATB1 proteins. After 24 to 48 hr, cells grown on glass coverslips were fixed in paraformaldehyde, stained with DAPI and viewed under a fluorescence microscope at two different wavelengths.

3.3 FUNCTION OF SATB1 NUCLEAR MATRIX BINDING

Two different approaches were used to determine the function of the SATB1 NMTS between amino acids 224 and 228. First, SATB1 constructs lacking the NMTS(224-278) were transduced into cells carrying integrated MMTV proviruses or integrated MMTV LTR-reporter constructs. These experiments were used to test whether the NMTS is critical for the ability of SATB1 to repress transcription from the MMTV LTR. Second, because the NMTS likely interacts with specific cellular proteins to exert its function, the NMTS was used as bait in the yeast two-hybrid assay.

3.3.1. Nuclear matrix binding regulates SATB1-mediated transcriptional repression.

Previous data indicated that specific SATB1 suppression of MMTV transcription was demonstrable on integrated, but not transiently transfected, constructs containing the MMTV LTR (Fig. 16, 22, and 23). Thus, stably integrated proviruses in MMTV-infected XC cells (XC/MMTV) were used to assess the functional effect of NMTS deletion. MigR1, MigR1-SATB1, and MigR1-ΔNMTS [previously known as ID(224-278)] vectors were independently co-transfected into 293T packaging cells with plasmids capable of expressing *gag-pol* and VSV G protein. The infectious particles were concentrated, tittered, and. XC/MMTV cells were transduced with equal numbers of infectious particles containing the empty vector or vectors containing either wild-type SATB1 or SATB1ΔNMTS. Cells then were expanded and induced with DEX for 24 hr to maximize MMTV expression from the LTR.

Since the MMTV LTR promoter directs expression of the *gag-pol* mRNA, Western blotting was performed to compare the relative expression of MMTV Gag protein (Fig. 35). Expression of wild-type SATB1 significantly suppressed Gag expression compared to that produced in the presence of the MigR1 control vector (compare lanes 2 and 3, upper panel). Deletion of the NMTS relieved this suppression. Western blotting also revealed that comparable amounts of wild-type and mutant SATB1 were expressed (Fig. 35, middle panel). Actin-specific antibody showed that similar amounts of protein were loaded in each lane of the gel (lower panel).

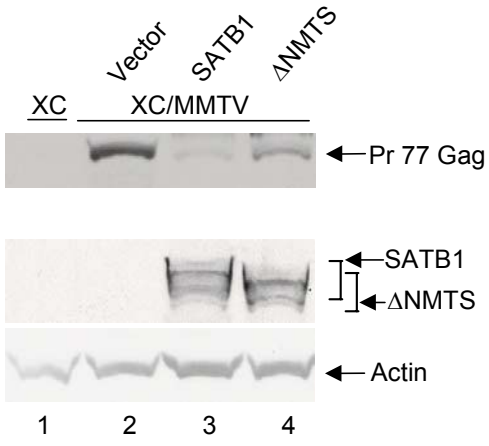


Fig. 35. Deletion of the NMTS relieves SATB1-mediated repression of Gag expression from integrated MMTV proviruses.

XC/MMTV cells were incubated for 4 to 5 days post-transduction (MOI=1) and then incubated in the presence of 10^{-6} M DEX for an additional 24 hr. The transduction efficiency was >95% as determined by FACS analysis. Protein extracts were subjected to Western blotting, and blots were incubated with antibodies specific for MMTV Gag (upper panel), SATB1 (middle panel), or actin (lower panel).

Because Western blotting is only semi-quantitative, RNase protection assays (RPAs) were used to quantitate the effect of NMTS removal on SATB1-specific repression. Again, XC/MMTV cells were transduced with equal amounts of infectious particles from MigR1, MigR1-SATB1, and MigR1-ΔNMTS. Over 95% of the cells were transduced as determined by FACS analysis. After establishment of the viral infection, cells were treated with DEX for 24 hr to induce MMTV expression, and then total RNA was extracted. RPAs were performed using labeled MMTV LTR and *gapdh* probes, and MMTV RNA levels were quantitated using a phosphorimager after normalization for *gapdh* expression (Fig. 36). Results indicated that SATB1 overexpression reduced MMTV transcription by approximately four-fold, whereas the SATB1ΔNMTS gave ca. three-fold abrogation of this effect (compares lanes 4-6).

A similar experiment was performed using rat XC cells that had stably integrated copies of an MMTV LTR-luciferase reporter gene (XC/MTV-LUC). Stably transfected cells were transduced with equal numbers of infectious particles containing the MigR1, MigR1-SATB1, MigR1-ΔNMTS, or MigR1-ΔDBD [also known as ID(346-494)] vectors as described previously. Transduced cells then were treated with or without DEX for 24 hr, and then protein extracts were prepared. Western blotting confirmed similar expression levels of wild-type and mutant SATB1 proteins (Fig. 37A). Luciferase assays indicated that wild-type SATB1 expression reduced LTR-directed reporter gene expression 5.5-fold in the presence, but not the absence, of DEX (Fig. 37B). Deletion of either the NMTS or the MAR-binding domain (DBD) significantly relieved this suppression.

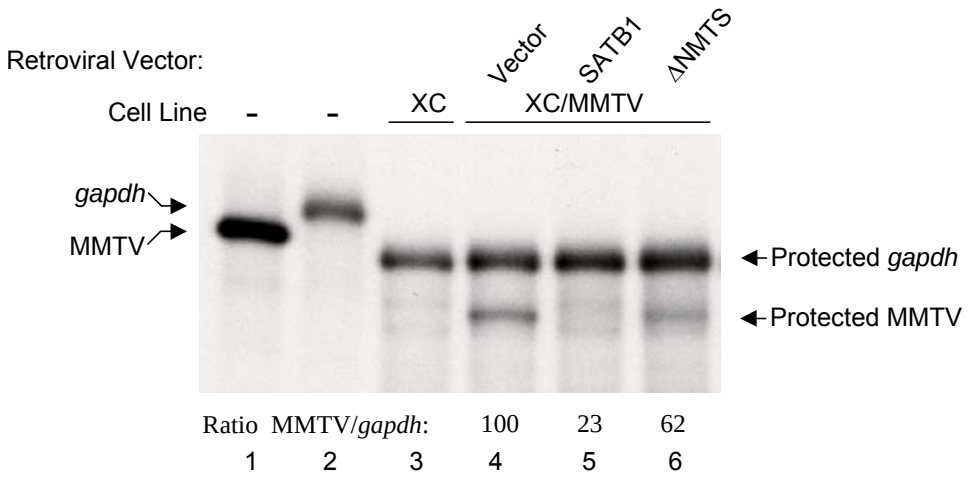


Fig. 36. Deletion of the NMTS relieves SATB1-mediated repression of the MMTV LTR in XC cells in RNase protection assays.

XC/MMTV cells were transduced with MigR1, MigR1-SATB1, or MigR1-ΔNMTS. Four to five days post-infection, 10^{-6} M DEX was added for 24 hr, and total RNA was extracted for RPAs. RNAs were hybridized to labeled probes specific for the MMTV LTR and *gapdh*. MMTV expression was quantitated using the phosphorimager and normalized to *gapdh* levels. Bands proximal to MMTV-specific bands represent incompletely digested probe since they also appear in reactions containing RNA from uninfected XC cells.

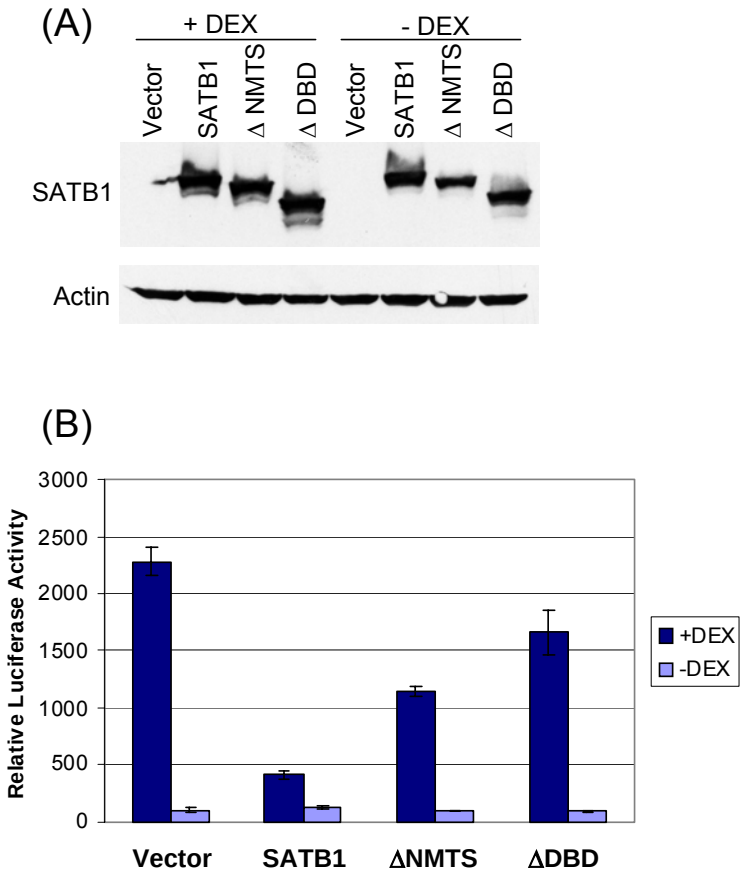


Fig. 37. The NMTS is required for optimal SATB1-mediated repression of the MMTV LTR in the presence, but not the absence, of glucocorticoids.

(A) Western blotting of wild-type and SATB1 mutants in XC cells expressing a stably integrated MMTV LTR-luciferase construct. Western blots were incubated with SATB1- or actin-specific antibodies (upper and lower panels, respectively). (B) Reporter gene assays of XC/MTV-LUC cells expressing wild-type or mutant SATB1 proteins. The luciferase activity of the same amount of protein from each sample was normalized to that of cells transduced with the MigR1 control virus (assigned a relative value of 100) (MOI=15). Data are expressed as an average of three independent luciferase assays from the same transduction, and error bars represent the standard deviations from the means. The luciferase activity of extracts from SATB1-transduced cells was significantly different ($P<0.01$; two-tailed Student's t test) from that obtained with vector-transduced cells. Results from ΔNMTS or ΔDBD-transduced cells also were significantly different from results obtained with SATB1-transduced cells.

Another cell line, HC11 mammary cells stably transfected with MMTV-LUC (HC11/MTV-LUC) were used to evaluate the effect of the NMTS of SATB1-mediated repression. HC11/MTV-LUC cells were transduced with the same amounts of MigR1, MigR1-SATB1, MigR1-ΔDBD, and MigR1-ΔNMTS infectious particles. Approximately 90% of the cells were transduced. Following infection, cells were treated with and without DEX for 24 hr, and protein extracts were prepared. Similar amounts of the wild-type SATB1 and mutant proteins were produced as detected by Western blotting (Fig. 38A). SATB1 suppressed MMTV expression both in the presence and absence of glucocorticoids, and deletion of the NMTS partially relieved this suppression (Fig. 38B). As expected, deletion of the major SATB1 DNA-binding domain (MigR1-ΔDBD) completely abolished SATB1-mediated transcriptional repression from the MMTV LTR. Thus, the NMTS appears to be required for maximal effects of SATB1 on the MMTV LTR in the presence and absence of glucocorticoids.

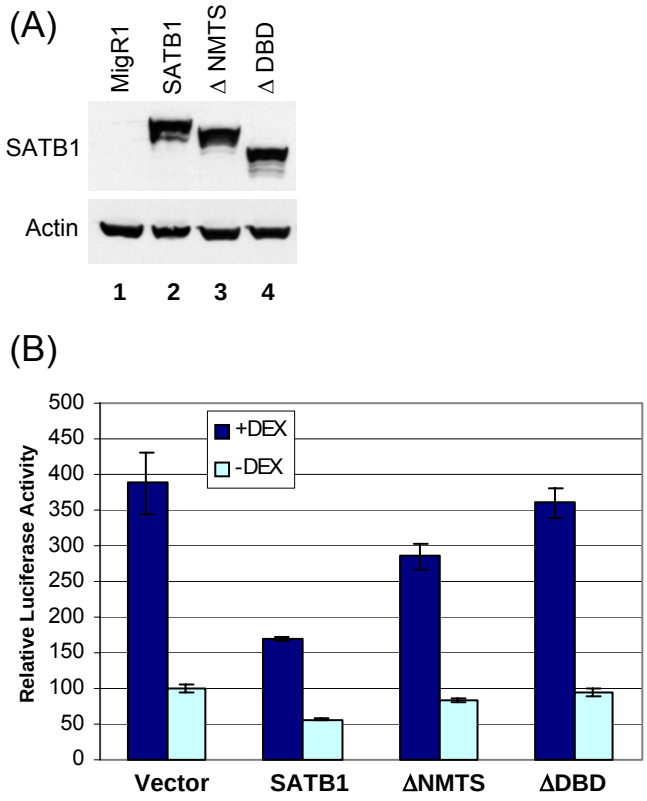


Fig. 38. Deletion of the NMTS relieves SATB1-mediated repression from the MMTV LTR in mammary cells.

(A) Western blot of wild-type and mutant SATB1 proteins in HC11 cells stably expressing MMTV-LUC. SATB1, ΔNMTS, and ΔDBD were ectopically expressed in HC11/MTV-LUC cells by transduction (MOI=15). Cells were then incubated for 24 hr in the presence or absence of 10^{-6} M DEX. Protein extracts were subjected to Western blotting and incubation in the presence of monoclonal SATB1-specific (upper panel) or actin-specific (lower panel) antibody. (B) Luciferase activity of stably integrated MMTV LTR-reporter genes in the presence of wild-type or mutant SATB1 proteins. HC11/MTV-LUC cells were treated as described in panel A. Luciferase values were calculated as described in Fig. 37. The luciferase activity of extracts from SATB1-transduced cells was significantly different ($P<0.01$; two-tailed Student's *t* test) from that obtained with control virus-transduced cells (either in the presence or absence of hormone). Results from MigR1-ΔNMTS and MigR1-ΔDBD-transduced cells (with or without hormone) also were significantly different from those obtained with similarly treated SATB1-transduced cells. The entire experiment was repeated with similar results.

SATB1 recruits chromatin remodeling complexes containing histone deacetylase 1 (HDAC1) to the IL-2Rα locus by directly interacting with HDAC1 (218). This result suggests that SATB1 might suppress IL-2Rα expression by modulating histone acetylation. Therefore, an HDAC inhibitor, trichostatin A (TSA), was used to test the involvement of HDAC1 on SATB1-mediated LTR repression. Previous data suggested that a low concentration of TSA activates stably integrated MMTV LTR expression, while higher concentrations inhibited MMTV transcription. HC11/MTV-LUC cells were transduced with MigR1, MigR1-SATB1, MigR1-ΔNMTS, or MigR1-ΔDBD infectious particles. Cells then were incubated without TSA or two different TSA concentrations for 24 hr. Interestingly, both concentrations of TSA inhibited MMTV expression (up to 2.5-fold) (Fig. 39), suggesting that increased acetylation of a cellular factor inhibits basal MMTV transcription. SATB1 expression reduced the TSA-mediated suppression. This effect appeared to be specific since the DBD-deletion mutant had similar luciferase values as vector-transduced cells. Surprisingly, NMTS deletion potentiated the effect of SATB1 on TSA-mediated suppression. These data suggest that SATB1 may directly or indirectly reduce acetylation of a factor(s) at the MMTV promoter, consistent with the ability of SATB1 to recruit HDACs (218). Deletion of the NMTS may potentiate HDAC recruitment.

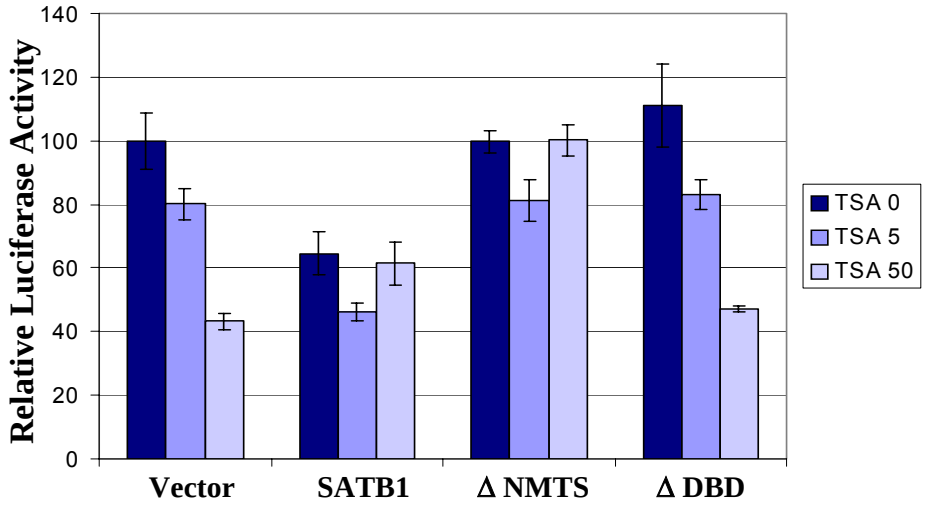


Fig. 39. Deletion of the NMTS potentiates the relief of TSA-mediated suppression of basal MMTV expression by SATB1.

HC11/MTV-LUC cells were transduced with equal amounts of MigR1, MigR1-SATB1, MigR1-ΔNMTS, or MigR1-ΔDBD infectious particles (MOI=15). Transduced cells were incubated without TSA or in the presence of 5 or 50 ng/ml TSA for 24 hr. Protein extracts were prepared, and equal amounts from each transduction were assayed in triplicate for luciferase activity. Luciferase activities were normalized to that of vector-transduced cells grown in the absence of TSA (assigned a value of 100). Cellular protein recoveries were similar in the presence or absence of TSA.

3.3.2. Cellular factors interacting with a SATB1 NMTS.

The previous section established that nuclear matrix binding is critical for SATB1-mediated transcriptional repression of the MMTV LTR. Since the SATB1 NMTS(224-278) is sufficient to direct fusion proteins to the nuclear matrix (Fig. 31-34), interaction with cellular proteins may be responsible for this effect. Therefore, a yeast two-hybrid screen was used to detect cellular proteins that interact with the SATB1

NMTS between amino acids 224 and 278. The NMTS was amplified by PCR and cloned into a GAL4 DBD-fusion vector (pGBKT7) to express Myc-tagged GAL4 DBD-NMTS in yeast. The yeast strain AH109 was then transformed with pGBKT7 or pGBKT7-NMTS, and protein expression was tested by Western blotting (Fig. 40). A construct expressing Myc-tagged GAL4 DBD-p53 protein was used as positive control. Each of the constructs produced a protein of the expected size.

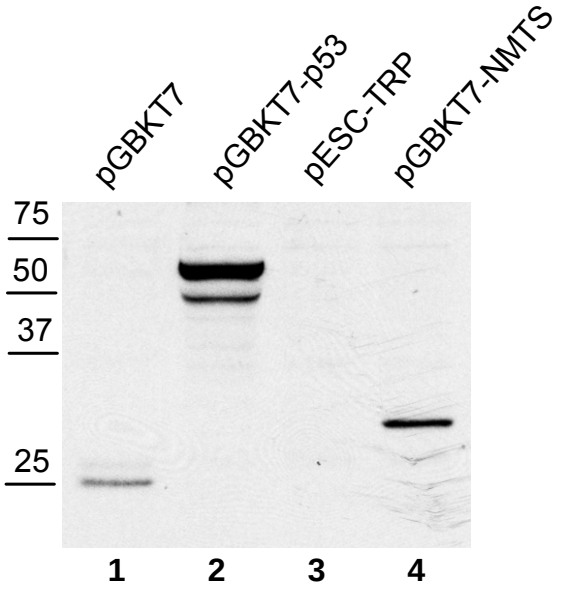


Fig. 40. GAL4-NMTS fusion protein is expressed in yeast cells.

Cell lysates were prepared from yeast cells transformed with constructs expresssing pGBKT7 (Myc-tagged GAL4 DBD), pGBKT7-p53 (Myc-tagged GAL4 DBD-p53), pESC-TRP (untagged GAL4 DBD), and pGBKT7-NMTS (Myc-tagged GAL4 DBD-SATB1 NMTS). Lysates were subjected to Western blotting and incubation with Myc-specific antibody. Untagged GAL4-DBD expression construct served as the negative control.

Subsequently, the GAL4 DBD and GAL4 DBD-NMTS expressing yeast cells were transformed with an expression library derived from human thymus cDNA fused to the GAL4 activation domain (AD). This library was chosen because of the high expression levels of SATB1 in thymus (53) and the known effects of SATB1 knockout on thymic gene expression (5). Cells were plated on three-dropout media plates (Leu-, Trp-, and His-) to select for retention of the GAL4 DBD and GAL AD plasmids as well as a reporter plasmid allowing *HIS3* synthesis. Since the reporter plasmid contains GAL4 DNA-binding sites upstream of the promoter, interaction of the GAL4 DBD and GAL4 AD on these sites should allow growth on three-dropout plates. Colonies were re-plated two more times on three-dropout media to remove false-positive colonies. Twenty six positive clones were selected and sequenced. Interestingly, one of the positive clones was lamin A, a major component of the nuclear matrix in some cell types (116) (Table 2). However, none of the clones were detected more than once, suggesting that the library screening had not been saturated.

Table 2. Clones selected during a yeast two-hybrid screen with GAL4-SATB1 NMTS.

A.

Gene Name	Frequency
3 BAC RP11-94E10	1
Actin, beta	1
Acyl coA synthetase 3	1
ADP-ribosylation-like factor 6 interacting protein 4	1

ADP-ribosyltransferase	1
APC11 anaphase promoting complex subunit 11	1
ATP synthase	1
Break point cluster region (BCR)	1
Chromosome 1 clone RP11-88N-11	1
Chromosome 17 clone hRPK, 481-C-4	1
Chromosome 5 clone CTC-453E13	1
Clathrin, heavy polypeptide	1
GlucosidaseI	1
Glyceraldehyde 3-phosphate-dehydrogenase	1
Heat shock 60kD protein 1	1
HS1 binding protein (HAX1)	1
Immunoglobulin heavy chain constant region gamma2	1
Lamin A	1
Nuclear transport factor 2 (NUTF2)	1
Protease subunit beta type 6	1
Ribosomal protein S20	1
Similar to unc-51-like kinase 2	1
Sn-RNP-specific protein, 116kD	1
Splicing factor SRp30C	1
Translation elongation factor 1 alpha 1	1
Tubulin, beta 5	1

B.

Gene Name	Frequency
A disintegrin and metalloproteinase domain 19 (meltrin beta) (ADAM19)	1
Actin, beta	1
FYN oncogene related to SRC, FGR, YES (FYN)	2
HLA-B associated transcript 3 (BAT3)	3
HSPC118	1
Human splicing factor SRp40-3	1
Koyt binding protein 1	1
Ubiquitin-conjugating enzyme, UBCH8	6

Two-step screens were used to identify weak interactions with the SATB1 NMTS. Thymic library transformants were plated on two-dropout selection media (Leu-, Trp-) to select transformants containing both a GAL4 DBD and a GAL4 AD. Cells then were plated on three-dropout media to identify plasmids that would allow synthesis of histidine. Sixteen clones were sequenced and identified (Rachel Misquitta, personal communication) (Table 2B), and UBCH8, BAT3, and FYN were identified more than once. No overlap was noted between the two screens.

Two clones expressing either UBCH8 or lamin A and a GAL4 DBD-NMTS subsequently were co-transformed. Assays for β-galactosidase activity suggested that

both candidate proteins interacted with the SATB1 NMTS (data not shown), and this interaction required the presence of both the AD and the DBD expression plasmids.

To verify the interaction between SATB1 and lamin A (Table 2A), a co-immunoprecipitation experiment was performed in 293T cells. Cells were co-transfected with expression constructs for wild-type SATB1 and Myc-tagged lamin A or Myc-tagged lamin C (Fig. 41). Lamin A and C differ only at their C-terminus (64). Cell lysates were incubated with antibody to Myc, and precipitates were analyzed by Western blotting and incubation with antibodies specific for Myc or SATB1. Myc-specific antibody co-precipitated SATB1 as well as both Myc-tagged proteins. SATB1 was not precipitated by Myc-specific antibody if Myc-tagged proteins were absent, although similar amounts of SATB1 were expressed in both cases.

Reciprocal experiments also were performed. GFP-SATB1 and either Myc-tagged Lamin A or Lamin C expression vectors were co-transfected into 293T cells. GFP-specific antibody co-precipitated both lamin A and lamin C, but only in the presence of SATB1 overexpression (Fig. 42). Interestingly, lamin C appeared to co-precipitate with SATB1 more efficiently than lamin A.

To determine the specificity of lamin A and C interaction with the NMTS, 293T cells were co-transfected with lamin A or C and ΔNMTS. Co-precipitation experiments indicated that SATB1 lacking the NMTS retained interaction with lamin A and C (Fig. 43). These data suggest that the interaction between lamins A/C and SATB1 is not specific to the NMTS.

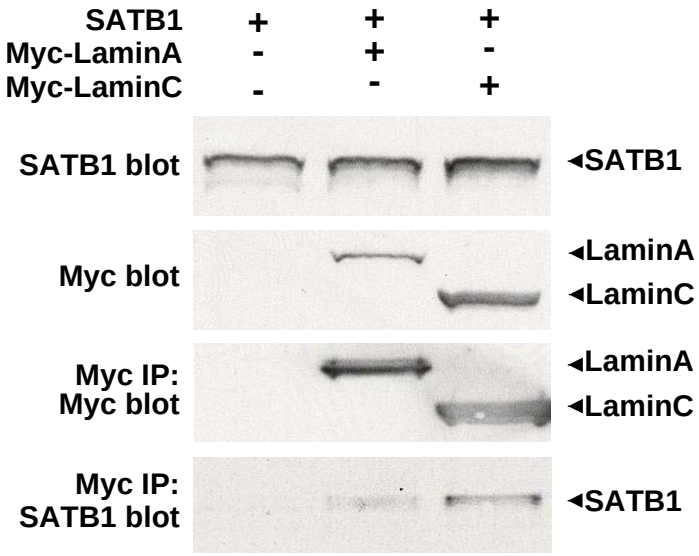


Fig. 41. Overexpression of SATB1 in 293T cells allows interaction with lamin A and C.

Myc-tagged lamin A or C expression plasmids were co-transfected with a SATB1-expression vector. Equal amounts of protein lysates were analyzed directly for protein expression by Western blotting with SATB1-specific or Myc-specific antibodies (upper two panels, respectively). Other portions of the same lysates were incubated with Myc-specific antibody, and precipitates were collected with protein A/G agarose, washed extensively, and analyzed on denaturing polyacrylamide gels. Western blots then were analyzed with Myc-specific or SATB1-specific antibodies (lower two panels, respectively).

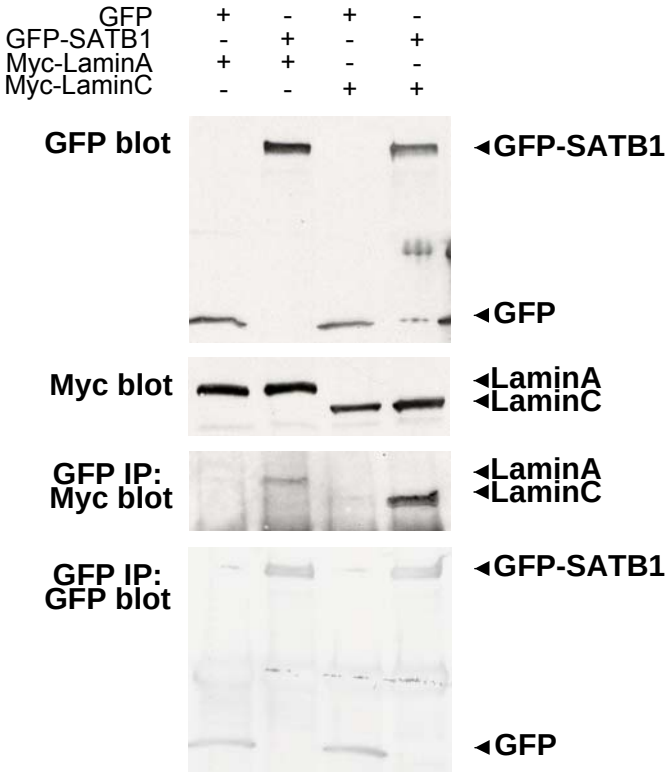


Fig. 42. GFP-tagged SATB1 co-precipitates with Myc-tagged lamin A and C.

GFP-SATB1 and Myc-tagged lamin A or C expression vectors were co-transfected into 293T cells. After 24 hr, transfected cells were lysed and analyzed directly by Western blotting with GFP-specific or Myc-specific antibodies (upper two panels, respectively). Another portion of the lysates was incubated with GFP-specific antibody and subjected to Western blotting with Myc-specific or GFP-specific antibodies (lower two panels, respectively).

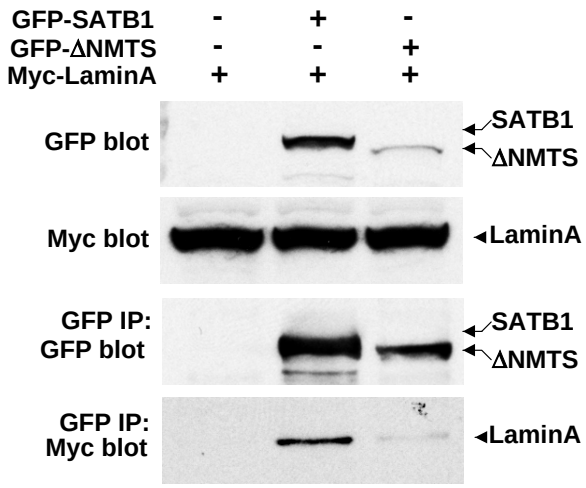


Fig. 43. Interaction between lamin A and SATB1 is not specific for the NMTS.

Myc-tagged lamin A expression plasmid was co-transfected with a GFP-SATB1 or GFP- ΔNMTSexpression vector. Equal amounts of protein lysates were analyzed directly for protein expression by Western blotting with GFP-specific or Myc-specific antibodies (upper two panels, respectively). Other portions of the same lysates were incubated with GFP-specific antibody, and precipitates were collected with protein A/G agarose, washed extensively, and analyzed on denaturing polyacrylamide gels. Western blots then were analyzed with Myc-specific or GFP-specific antibodies (lower two panels, respectively).

4. DISCUSSION

4.1 DEVELOPMENT OF AN MMTV REPORTER SYSTEM FOR SATB1 FUNCTION

SATB1 was cloned by screening a human testis cDNA library using the MAR sequence located 3' of the mouse immunoglobulin μ heavy chain (IgH) enhancer (53). SATB1 has been shown to regulate gene expression by binding to the matrix attachment regions (MARs) of various genes such as the DNase I-hypersensitive site (HS1) in the T cell receptor β locus, the gp91^{phox} promoter region, and the major breakpoint region (MBR) of BCL2 (38,80,172). Two groups have reported that SATB1 acts as a repressor, whereas others have suggested that SATB1 is an activator of gene expression (32,111,115,210). Subsequently, studies of SATB1-knockout mice revealed that 2% of 589 genes surveyed showed altered expression during T-cell development. RT-PCR analysis confirmed that 9 of 10 genes tested had increased expression in SATB1-null thymocytes, whereas one gene (*TDAG51*) had decreased RNA levels. These results suggest that SATB1 is primarily a repressor in the thymus (5). Thymocyte development was blocked mainly at the CD4+CD8+ stage, and homozygous knockouts died by 3 weeks of age (5).

Transcriptional studies of SATB1 have been particularly difficult because of problems associated with SATB1 overexpression and lack of a proper reporter system in cell culture (38,111). SATB1 has been reported to bind to MAR sequences within the MMTV NREs in the U3 region of the LTR (124). Mutation of a strong SATB1-binding site in the promoter-proximal NRE (+924) elevated transcription from the

MMTV standard LTR promoter using transient transfection assays, consistent with SATB1 as a transcriptional repressor of MMTV expression (25). Moreover, this same SATB1 mutation resulted in elevated expression from integrated LTR-reporter constructs in the lymphoid tissues of transgenic mice relative to mammary gland (124). Therefore, the well-characterized MMTV promoter was used to develop a reporter system for SATB1 repressor function in cell culture.

To develop an assay system that would depend on introduced SATB1 constructs, various cell lines were examined to determine their endogenous SATB1 level (Fig. 6). Several cell lines, including XC rat fibroblasts, mammary cells of mouse and human origin, and the BW5147 CD4+CD8+ mouse T cells were shown to have low SATB1 protein expression. Human breast cancer cells (MCF-7) initially were selected for the assay due to their low levels of endogenous SATB1 and their ability to support high levels of exogenous SATB1.

Initially, transient co-transfections were performed in MCF-7 cells with plasmids for wild-type SATB1 expression and an MMTV-luciferase reporter. Wild-type SATB1 suppressed the transiently transfected reporter plasmid up to ca. 10-fold in a dose-dependent manner (Fig. 12). Subsequently, various SATB1 mutants were constructed to map protein domains important for SATB1 transcriptional activity based on published results (Fig. 14) (52,68,149). Surprisingly the SATB1 mutant ID(346-494), which is missing the MAR DNA-binding domain, suppressed MMTV expression to levels similar to the wild-type protein (Fig. 16). Transcriptional repression of the MMTV LTR that is independent of DNA binding has been previously reported (39). Expression of a nuclear factor I (NFI-C) mutant, which lacks DNA-binding ability, suppressed MMTV

expression at wild-type levels, presumably by interference with coactivator function at the MMTV promoter (39). Testing of additional SATB1 mutants revealed that small fragments of both N- and C-terminus with no known function also suppressed MMTV expression about 4-fold. These results suggest that SATB1 may suppress MMTV expression from unintegrated DNA templates by squelching of general transcription factors. Thus, transient transfection assays do not appear to recapitulate SATB1-mediated repression of the MMTV LTR observed *in vivo*.

Transiently transfected and stably transfected plasmids carrying the MMTV LTR have been shown to have different chromatin structures in the nucleus. Binding of nuclear factor 1 (NF1) to the DNA, which is required for hormone-dependent chromatin remodeling and transcription activation, is distinct for unintegrated and integrated templates (83). NF1 binds constitutively to transiently introduced templates, whereas it is excluded from stably integrated templates until hormone treatment (7). Bovine papilloma virus (BPV)-based vectors are episomes that have a chromatin composition similar to stably transfected templates (189,200). Therefore, some studies of transcription from the MMTV LTR have been performed using BPV-based plasmids (36,157).

MMTV has a hormone-responsive element (HRE) in the LTR, and addition of glucocorticoids to virus-infected cells leads to induction of RNA expression by 10 to 50-fold (174). A bimodal mechanism has been proposed to explain MMTV promoter activation by steroid hormones (7). In the first step, the binding of ligand-bound hormone receptors induces changes in chromatin accessibility and facilitates NF1

binding. In a second step, the nuclear receptor interacts with additional transcription factors to recruit them to the promoter and further increase transcription (7).

Recently, three different forms of stably integrated MMTV promoters have been described: the transcriptionally inactive promoter, the basal promoter, and the hormone-activated promoter (69). Transcriptionally inactive promoters contain TBP and high levels of histone H1, whereas both the basal promoter and the glucocorticoid-activated promoter are depleted of histone H1 and contain RNA PolII, TBP, Oct1, and acetylated H3 tail domains. However, only the hormone-activated promoter contains stably bound BRG1 (69). These results and our experiments with transiently transfected MMTV LTR-reporter plasmids suggest that integrated transgenes will most closely mimick the function of SATB1 on integrated MMTV proviruses.

Rat fibroblast cells (XC), which express little endogenous SATB1 (Fig. 6), were selected to contain stably integrated MMTV LTR-luciferase plasmids. However, little effect was observed after transient transfection of SATB1 expression vectors using both lipid transfection protocols and electroporation (184). This result appears due to low transfection efficiency. Since the majority of the cells have stably integrated reporter genes and only a fraction of cells express SATB1, the effects of SATB1 overexpression could be easily missed because reporter gene activity from untransfected cells would mask that of transfected cells. Therefore, a retroviral expression system was adopted to express SATB1 in >90% of the cell population. The retrovirus-based expression assays unequivocally demonstrated that SATB1 suppresses stably integrated MMTV expression in several different cell types (Figs. 17-23).

These experiments also showed that SATB1 suppresses MMTV expression in the absence as well as the presence of the hormone, dexamethasone (DEX), a synthetic glucocorticoid (Figs.13 and 21). Interestingly, transiently transfected and stably integrated reporter genes showed different responses to DEX induction in rat XC cells. SATB1 suppressed the transiently transfected reporter gene both in the presence and in the absence of DEX (Fig. 13). However, in stably transfected XC cells, SATB1 suppressed the expression of the MMTV reporter gene in the presence, but not in the absence, of DEX (Fig.17-20).

The result that SATB1 suppressed stably transfected MMTV reporter genes only in the presence of hormone induction was unexpected. Therefore, two other cell lines, mouse mammary HC11 cells and mouse BW5147 T cells were tested to determine whether hormone induction was required for SATB1-mediated suppression of the stably transfected reporter gene. Two cell lines showed that SATB1 represses MMTV reporter gene both in the presence and absence of hormone induction (Fig. 21). The result observed in XC cells may be cell line-specific for several reasons. First, mice carrying MMTV LTRs with SATB1-binding site mutations showed high reporter gene expression in thymus in the absence of hormone induction (124). CCAAT displacement protein (CDP), which has similar protein domains and also binds to the MMTV NRE, suppresses MMTV expression both in the presence and absence of hormone induction (226). Finally, knockdown of SATB1 expression in Jurkat cells, which express high endogenous SATB1, showed elevation of MMTV expression in the absence of glucocorticoid induction (Fig. 24).

The observation that hormone induction is required for SATB1-mediated suppression in XC cells is interesting. *In vivo* SATB1 binding to the MMTV promoter may increase in the presence of hormone induction in XC cells, and therefore, our ability to detect SATB1-mediated suppression may be enhanced. Transcription factors, such as NF1 and Oct1, bind to stably integrated MMTV promoter in the presence of hormone induction after the chromatin structure of the stably integrated MMTV gene is remodeled by a hormone-nuclear receptor complex (7,69). This result suggests that chromatin immunoprecipitation (ChIP) using SATB1 antibody would precipitate more MMTV fragments in the presence of hormone induction. Additionally, restriction enzyme accessibility assays (65) should reveal a relaxed chromatin structure on the SATB1 binding sites after hormone induction. Therefore, ChIP experiments and restriction enzyme accessibility assays may clarify the relationship between SATB1-mediated suppression and MMTV chromatin structure in XC cells and other cell lines.

Why then does SATB1, a repressor, bind to the MMTV LTR when the promoter is activated by a hormone-receptor complex? This dilemma can be explained through an understanding of the tissue distribution of SATB1 and the MMTV life cycle. SATB1 is primarily expressed in the thymus (53), with little expression in other tissues, including mammary glands. Milk-borne MMTV infects B- and T-lymphocytes, which then deliver the virus to the mammary gland, the final destination. Despite the early infection of lymphoid cells with MMTV, the virus induces breast cancer, not lymphomas. However, MMTV variants that cause thymic lymphomas contain a 300-500 bp deletion in LTR U3 region (225), which binds at least two cellular proteins, SATB1 and CDP. Since CDP is ubiquitously expressed, SATB1 is a good candidate for the suppression

of MMTV expression in T cells to prevent the development of lymphomas. In support of this idea, as previously mentioned, mutation of a strong SATB1-binding site in the MMTV LTR elevated transgene expression in lymphoid tissues relative to mammary tissues (124).

Hormones have been shown to influence the development of mammary tumors in female mice. Tumor incidence is dramatically increased by hormonal stimulation in BALB/c mice infected by foster nursing on MMTV-infected C3H mothers (135). Mammary tumor incidence and onset dates also were increased by hormonal stimulation in the RIII strain (191). The increased mammary tumor incidence and/or the decrease in the mean latent period for tumor development by hormonal stimulation is observed in most of strains of mice (135,191).

MMTV is well adapted to spread vertically from infected mice to progeny. The virus initially infects lymphocytes, which then deliver virus to mammary glands. The viral production is suppressed in the mammary glands until the mice nurse their pups, thus achieving maximal virus concentration in the milk during lactation. Lymphoma development prior to nursing the pups would be disadvantageous for vertical transmission of the virus. MMTV does not encode an oncogene and induces tumors by insertional activation of oncogenes. Random insertional mutagenesis is increased by hormone induction of MMTV transcription, resulting in mammary tumors. Therefore, MMTV may not cause lymphomas until transcription levels are sufficiently elevated by hormone induction. Increased transcriptional levels also result in multiple rounds of reverse transcription, which produce mutations in the MMTV genome. Some of these mutations lead to the appearance of altered enhancer sequences within the LTR.

Insertion of a T-cell specific enhancer into MMTV LTR dramatically increased transcription of a reporter gene in transient assays. However, deletion of the NRE from the MMTV LTR marginally increased reporter gene activity in these assays (137). Substitution of U3 sequences from the MMTV LTR with those from TBLV, a lymphomagenic variant, resulted in viruses that induced exclusively T-cell lymphomas (137,213). Similarly, the contribution of NRE deletion and T-cell enhancers for tumor development was tested by injections into mice of cells expressing MMTV proviruses with an NRE deletion or insertions of a T-cell specific enhancer (20). Proviruses with the NRE deletion alone induced sporadic mammary tumors, whereas MMTV proviruses carrying a T-cell enhancer upstream of the NRE developed both T-cell lymphomas and mammary tumors. Interestingly, Southern blot analysis of DNA extracted from lymphomas, but not mammary tumors, revealed loss of the NRE from integrated MMTV proviruses (20). These results suggest that acquisition of a T-cell specific enhancer and loss of the NRE with its SATB1-binding sites both are necessary for MMTV induction of T-cell lymphomas (20).

CDP also has been shown to bind the MMTV NRE and is expressed in the thymus (124). CDP suppressed co-transfected MMTV reporter gene activity in a dose-dependent manner, and CDP-binding site mutations in the MMTV LTR relieved this suppression (227). Furthermore, CDP suppressed MMTV expression in the absence as well as in the presence of hormone induction (226). Although both SATB1 and CDP reduce MMTV expression in the presence of hormones (226), glucocorticoids result in 10- to 50-fold activation of viral transcription. Thus, the combined action of the two

suppressors may be required to reduce hormone-activated MMTV expression in lymphocytes.

A 150kD truncated CDP appears in lactating mammary glands of mice, and the truncated form does not bind to the MMTV NRE (227). Therefore, MMTV expression is maximal in the mammary glands during lactation during glucocorticoid induction after this repressor loses its function. It would be interesting to determine if CDP or SATB1 truncation also occurs in the thymus during T-cell development to allow higher MMTV transcription after lymphoid differentiation.

SATB1 may be a key regulator to suppress hormone-induced MMTV expression in lymphocytes. If this is the case, SATB1-null mice (5) should develop lymphomas as well as mammary tumors when the null mice are infected with MMTV. However, since SATB1-null mice die within 3 weeks of age, this experiment is not feasible (5). SATB1 suppresses MMTV expression in a dose-dependent manner (Fig. 12 and 18). Therefore, heterozygous mice, which have half the normal levels of SATB1 in thymi, might be used for this experiment (5). The SATB1-heterozygous mice may develop T-cell lymphoma due to high levels of MMTV insertional mutagenesis in the thymus more frequently than wild-type littermates. On the other hand, tumorigenesis by TBLV, which lacks SATB1-binding sites in the LTR, should be unaffected in SATB1 heterozygotes.

What is the molecular mechanism involved in the suppression of hormone-induced MMTV expression by SATB1? It was recently reported that SATB1 binds to p300 (66) and the p300-SATB1 complex appears to bind to the MMTV NREs (184). CBP and p300 are transcriptional co-activator proteins that act as transcriptional

bridges and scaffolds (35). Thereby, they mediate interactions between DNA-binding transcription factors and components of the basal transcription machinery (96). CBP/p300 binding to nuclear receptors is necessary for receptor function (45,100) perhaps providing a clue to the mechanism for repression of hormone-induced MMTV expression by SATB1.

The integrated MMTV promoter is organized as a phased array of six nucleosomes (Fig. 3), which interferes with NF1 and OTF1 binding. One model predicts that SATB1 binding to the MMTV NRE increases following hormone induction (Fig. 44). Once nuclear hormone receptor binds to the HRE, GR recruits the BRG1 complex and activators such as p300/CBP (82). The BRG1 complex then alters MMTV promoter structure so that H1 dissociates from the promoter, and NF1 and OTF1 are recruited. SATB1 binding to the promoter also increases, thus leading to additional interactions with p300 (Fig. 44) and fewer interactions with other transcription factors, including ligand-bound hormone receptor.

In this model, the overexpression of p300 or GR would abrogate suppression by SATB1 of the MMTV promoter. Indeed, overexpression of GR or p300 alleviates NF1-C-mediated repression of MMTV in the presence of hormone induction in HeLa cells (39). As the model predicts, CBP/p300 is present in limited concentrations inside the cells (86,165). In agreement with this observation, the Rubinstein-Taybi syndrome, results in severe developmental abnormalities from the loss of a single CBP allele (165). Further demonstration of the key role of CBP/p300 involve the ability of interferon-α to inhibit TNFα-stimulated HIV expression. This inhibition is achieved by

the competition between STAT-2, which is stimulated by IFN-α and RelA, and TNFα for interactions with p300 (86).

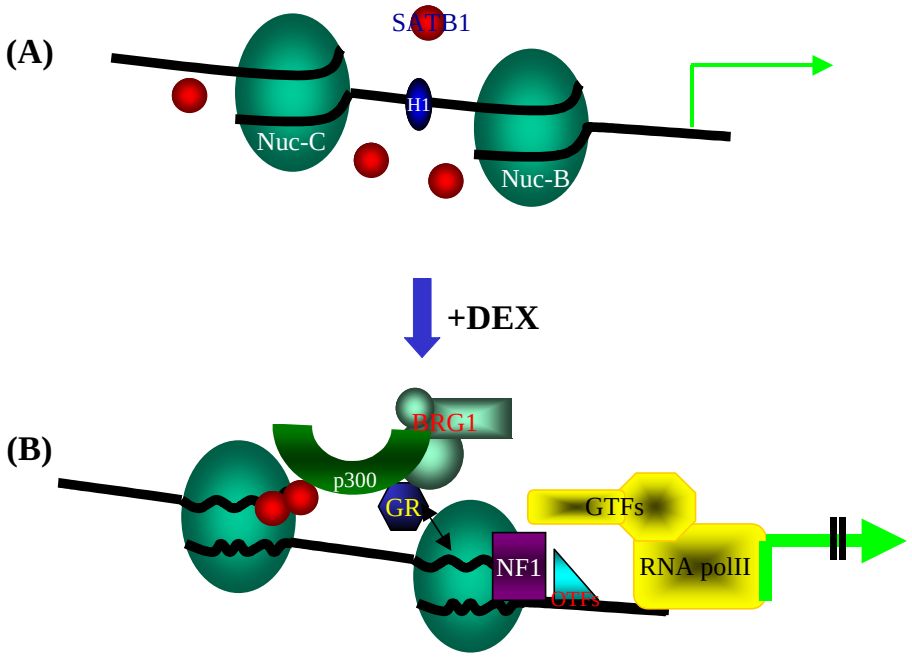


Fig. 44. Model for SATB1-mediated repression of glucocorticoid-induced transcription from the MMTV LTR.

(A) The integrated MMTV LTR is organized as a phased array of six nucleosomes. SATB1-binding sites in the NRE are tightly arranged by Nuc-C, limiting SATB1 binding to the promoter. (B) When glucocorticoid receptor binds to DEX, the complex enters into the nucleus and binds to Nuc-B, resulting in chromatin remodeling by the BRG1 complex. NF1 and OTF1 are recruited, and the binding of general transcription factors, activators, and RNA pol II is stabilized, resulting in increased MMTV transcription. Additionally, the efficiency of SATB1 binding to Nuc-C is increased by ligand-bound GR, allowing greater SATB1 interactions with p300 and fewer interactions with GTFs and/or the BRG1 complex. Abbreviations: Histone 1 (H1), Nucleosome B, C-(Nuc-B, C), dexamethasone (DEX), Glucocorticoid receptor (GR), Nuclear factor I (NF1), OTFs (Octamer transcription factors), BRG1 (Brahma-related gene 1), General transcription factors (GTFs), and RNA polymerase II (RNA pol II).

4.2. MAPPING AND FUNCTION OF SATB1 NUCLEAR MATRIX TARGETING SEQUENCE

SATB1 was originally identified as a thymus-specific transcription factor that bound to DNA associated with the nuclear matrix (i.e., MARs) (53). The nuclear matrix is the residual nuclear structure remaining after extensive extraction using treatments with a detergent, DNase, and high salt. The nuclear matrix serves as an attachment site to form loop structures of chromatin and plays a pivotal role in nuclear metabolism, e.g., DNA replication, transcription, and splicing. A nuclear matrix targeting sequence (NMTS) is responsible for association of proteins with the nuclear matrix. Sequences mediating nuclear matrix association have been determined in a number of proteins, including AML-1B, regulator of G protein signaling (RGS12TS-S), Runx2/Cbfa1, human nuclear protein NXP-2, the C-terminal domain of Ying Yang 1 (YY1), human glucocorticoid receptor, and human androgen receptor (29,37,106,134,205,220,223). In addition, the structure of an AML-1 NMTS of 31 amino acids was determined by X-ray crystallography and consisted of two loops connected by a flexible U-shaped peptide chain (197).

Since SATB1 associates with the nuclear matrix (50), a series of SATB1 deletion mutations were constructed to delineate the NMTS (Fig. 14). Using biochemical fractionation of transiently transfected cells, an NMTS was mapped to the 55 amino acids between the dimerization and MAR domains (residues 224 to 278) (Fig. 29). An internal deletion mutant of SATB1 lacking the NMTS [ΔNMTS or ID(224-278)] significantly reduced nuclear matrix association. However, since this deletion did not completely abolish the association, SATB1 may have more than one domain that

specifies matrix binding. Specifically, ID(346-494), a SATB1 mutant missing the MAR-binding domain, also significantly reduced nuclear matrix association, suggesting another NMTS in the DNA binding domain (Fig. 29). However, this mutant did not bind to the MMTV LTR, preventing separation of the functional effects of DNA binding and nuclear matrix binding on MMTV transcription. Therefore, the NMTS within the ID(346-494) mutant was not studied further. Nevertheless, fusions of the NMTS(346-494) to the GAL4 DNA-binding domain could be used to measure the effects of nuclear matrix binding with a GAL4-responsive reporter vector (37).

The presence of multiple NMTSs has been observed in steroid hormone receptors, AML1/ETO, ETO, and YY1 (12). The results of SATB1 mutant localization experiments are consistent with the idea that different proteins may act as receptors for directing proteins to specific subnuclear domains. For example, the nuclear protein RGS12TS-S has different targeting sequences for the nuclear matrix and subnuclear dots (37). Lamin B, a known component of the nuclear matrix, clearly has a different nuclear distribution than SATB1 (Fig. 27). Moreover, the SATB1 NMTS(224-278) showed minimal sequence homology with the NMTS of Runx family proteins (Fig. 45). Thus, there may be separate subdomains within the nuclear matrix, which may explain the lack of a consensus NMTS (37) (Fig. 45).

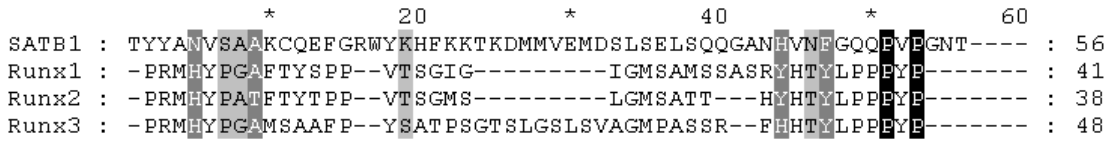


Fig. 45. Alignment of the nuclear matrix targeting sequence of SATB1 and mouse Runx family proteins.

Amino acids sequence alignments of the NMTSs of mouse Runx family proteins (220) and SATB1 were performed using CLUSTALW and graphically delineated using the GeneDoc software. The numbers at the right side indicate the number of amino acids. Amino acids are highlighted with black (a fully conserved residue), dark gray (a strongly conserved residue), and light gray (a weakly conserved residue).

The presence of an NMTS within the SATB1 N-terminus was confirmed by tethering amino acids 224 to 278 to either Lex A or GFP, which do not normally bind the nuclear matrix. Both GFP-NMTS and LexA-NMTS partially relocalized to the nuclear matrix (Fig. 31). Interestingly, the SATB1 NMTS also partially redirected GFP to the chromatin fraction. This result may indicate that the 55 amino acids designated as the SATB1 NMTS could be further delimited to give either nuclear matrix or chromatin binding. In addition, the SATB1 mutant (1-494) containing the PDZ dimerization domain, the NMTS, and the MAR-binding domain localized entirely to the chromatin and nuclear matrix fractions, whereas SATB1(1-224) containing only the PDZ domain was exclusive to the soluble nuclear fraction (Fig. 29). SATB1 localization to both fractions decreased after deletion of all or part of the MAR domain in various mutants (Fig. 29). Although localization in the soluble fraction may indicate the lack of

specific intranuclear trafficking signals, it is also possible that the presence of both the MAR and NMTS domains increases the affinity for both the nuclear matrix and chromatin. Previous data have shown that GR association with the nuclear matrix is increased by the presence of the GR DNA-binding domain (199). SATB1 domains present at the N-terminus or C-terminus may regulate both chromatin and nuclear matrix association.

The nuclear matrix, a subnuclear domain, plays an important role in transcriptional regulation (40,209). Therefore, the effect of nuclear matrix association on the ability of SATB1 to repress MMTV transcription was determined. Deletion of the NMTS(224-278) relieved SATB1-mediated repression of transcription from integrated MMTV proviruses or MMTV-LTR reporter plasmids (Figs. 35-38), but there was no detectable effect using transiently transfected plasmids containing the MMTV LTR (Fig. 16). These observations are reminiscent of the results of Kohwi-Shigematsu et al. in which SATB1 suppressed the expression of stably transfected, but not transiently transfected, MAR constructs (111). Although reduced suppression by ΔNMTS [also known as ID(224-278)] may be due to conformational changes within the protein, this mutant maintained several functional properties, including nuclear import and DNA binding (Figs. 25 and Data not shown). Partial relief of suppression after NMTS deletion might result from the retention of other nuclear matrix association sequences. Repression by SATB1 and the effect of NMTS(224-278) deletion also were independent of the cell type examined since similar results were obtained using mouse mammary gland and rat fibroblast cell lines (Figs. 35-38). Previous data have shown that MAR binding to the nuclear matrix is independent of cell type (124,208).

Nuclear matrix binding clearly has functional implications for transcription. Fractionation studies show that transcriptionally active DNA is tightly associated with the nuclear matrix, whereas inactive loci are not. For example, the ovalbumin gene in the chicken oviduct is associated with the nuclear matrix when the gene is stimulated by steroid hormones. However, when the hormone is withdrawn, the gene is detached from the nuclear matrix (42). Genes expressed by integrated polyoma or avian sarcoma viruses in transformed cells also were associated with the nuclear matrix, but subclones that lack the transformed phenotype and express no detectable viral transcripts show little association with the nuclear matrix (46). Mutations of Pit-1, a transcriptional activator of prolactin, altered nuclear matrix localization and led to dwarfism in mice (129). Knock-in mice that express an NMTS-deleted form of Runx2 have abnormal bone formation and die during embryonic development (40). The Runx2 NMTS autonomously exhibits transactivation in a mammalian one-hybrid system (220), whereas deletion of the Runx2 NMTS abrogated the transactivation effect on the bone-specific promoters, osteocalcin and transforming growth factor β R1 (40,187,220). An NMTS-deletion mutant of Hic-5/ARA55, a GR coactivator, maintained its GR-binding ability in yeast cells, but did not fully activate a glucocorticoid-responsive promoter (78). NMTS deletion from transcription regulators also affects repression of certain promoters. RGS12TS-S inhibits transcription *in situ* and in a mammalian one-hybrid assay, and NMTS deletion relieved this suppression (37). Removal of the Runx2 NMTS abrogated repression of the bone sialoprotein promoter (40). Therefore, the nuclear matrix affects gene activation as well as repression.

SATB1 suppressed integrated reporter gene expression in the presence and absence of ligand-bound GR (Fig. 38). GR functions by recruitment of co-activators that acetylate histones and chromatin remodeling complexes (122), and the subsequent chromatin reorganization allows promoter-proximal NF1 and Oct1 binding and assembly of the basal transcription machinery (14,148). Both GR and SATB1 are bound to the nuclear matrix (50,199), where there may be competition between recruitment of HATs and HDACs. Such competition may explain the partial effect of SATB1 overexpression on hormone-induced MMTV expression. NMTS deletion would decrease SATB1-mediated HDAC recruitment, which may impede interaction with CHRAC, ACF and NURD chromatin remodeling complexes (218). SATB1 is bound to MARs at the bases of chromatin loops (50) and may regulate effects of *cis*-acting regulatory elements in cellular DNA by acting as an insulator (151). For MMTV, which contains MARs at either end of the genome in the LTR (124), insulation from surrounding cellular regulatory control after genome insertion may provide repression of viral transcription in T cells and a selective advantage for virus survival.

SATB1 is known to recruit chromatin remodeling complexes, which contain HDAC-1 and -2, to change overall loop structures of chromatin (218). SATB1 binds to the promoters of IL-2 and IL-2Rα and recruits HDAC1, which results in decreased levels of transcription (115). Thus, SATB1 may repress MMTV expression by HDAC recruitment to the MMTV LTR. This hypothesis was tested using a specific HDAC inhibitor, trichostatin A (TSA).

Acetylation of histones has long been correlated with transcriptional activity (4). Generally, acetylation of the N-terminal tails of the core histones opens the

nucleosome structure and enhances transcription, whereas deacetylation has the opposite effect, mediating transcriptional suppression (3,26,99,156). However, the relationship between histone acetylation and transcription is more complicated (34,51,180). For example, in mammalian cells, c-*myc* and cyclin D1 genes are repressed by HDAC inhibitors (112,185). Interestingly, MMTV transcription was both enhanced and reduced by the addition of TSA, depending on the concentration of the HDAC inhibitor (13).

Two different concentrations of TSA were tested to determine whether the effect on SATB1-mediated MMTV repression. Both low and high concentrations of TSA suppressed luciferase activity driven by the MMTV promoter in HC11 mammary cells (Fig. 39). However, SATB1 and ΔNMTS [ID(224-278)] nullified TSA-mediated repression of MMTV transcription at high concentrations of TSA, while ΔDBD and empty vector did not affect MMTV expression (Fig. 39). These results suggest that deletion of nuclear matrix association sequences between amino acids 224 and 278 has no effect on HDAC association with SATB1.

A previous report showed that TSA-mediated repression was mediated through the TATA box region and was independent of chromatin remodeling and histone acetylation. These results suggest that HDAC inhibitors may affect acetylation of non-histone proteins (145). Thus, SATB1 binding to DNA also may determine the function of unidentified non-histone proteins in the TATA region of the MMTV LTR.

It appears likely that the function of the SATB1 NMTS(224-278) is mediated through functional interaction with other nuclear proteins. A yeast two-hybrid screen was used to identify proteins that interact with this NMTS in the nuclear matrix (Fig. 40).

Two different screens identified about 40 proteins (Table 2) as potential interaction partners of NMTS(224-278). Lamin A, which is one of the major nuclear matrix proteins, was identified through the screen and the interaction between lamin A and the NMTS was confirmed using co-immunoprecipitation experiments with full-length SATB1 and lamin A (Figs. 41 and 42). However, co-immunoprecipitation experiments with wild-type SATB1 and ΔNMTS showed no significant binding specificity between lamin A and the NMTS(224-278) since both wild-type SATB1 and ΔNMTS interact with lamin A in 293T cells (Fig. 43).

Two major types of lamins are found in most mammalian cells: B-type lamins (B1 and B2), which are found in all somatic cells, and A-type lamins (A and C), which are expressed primarily in differentiated cells (175,196). Lamin A was overexpressed in XC cells carrying integrated MMTV LTR-reporter plasmids to determine its effect on SATB1-mediated repression of viral transcription. However, neither negative nor positive effects on MMTV transcription were observed (184). Since lymphocytes do not usually express A-type lamins, (171,179,184), the interaction between lamin A and SATB1 may play a more important role in other tissues, such as brain or testis.

It is still not clear how nuclear matrix binding regulates SATB1-mediated repression of MMTV RNA levels. However, AML1 and the corepressor mSin3A are colocalized in the nuclear matrix when AML1 is not phosphorylated (92). Upon phosphorylation, AML1 translocates from the nuclear matrix, is released from mSin3A, and transcriptional activity is enhanced (92). This phosphorylated form of AML1 is subject to proteasome-mediated degradation in a time-dependent manner (92).

SATB1 also has been reported to be phosphorylated, although direct evidence for this modification is lacking (68).

Recently, SATB1 was shown to be sumoylated (72). SUMO modification is known to regulate activity and/or nuclear and subnuclear localization of many proteins, including Sp3, RanGAP, and PML (71,131,176,224). Therefore, the activity and/or nuclear matrix localization of SATB1 may be modulated by sumoylation. Identification of proteins that interact with SATB1 in the nuclear matrix as well as SATB1 modifications that influence protein-protein interactions should enhance our understanding of the mechanisms by which SATB1 binding to the nuclear matrix regulates MMTV expression. In addition, these studies should illuminate the larger questions regarding the role of the nuclear matrix in eukaryotic transcriptional regulation.

APPENDIX

List of abbreviations used:

ACF	ATP-utilizing chromatin assembly and remodeling factorY
AML	acute monocytic leukemia
BRG1	Brahma-related gene 1
Bright	B cell regulator of IgH transcription
BURs	base-unpairing regions
CAT	chloramphenicol acetyltransferase
CBP	CREB-binding protein
CDP	CCAAT-displacement protein
CH	Chromatin fraction
ChIP	chromatin immunoprecipitation
CHRAC	chromatin-accessibility complex
CR	Cut-like repeats
CREB	cAMP responsive element binding protein
DAPI	4',6-diamidino-2-phenylindole
DBD	DNA-binding domain
DEX	dexamethasone
DMEM	Dulbecco's modified Eagle's medium
DOX	doxycycline
DRIP	vitamin-D-receptor interacting protein
EGF	epidermal growth factor
EMSA	electrophoretic mobility shift assay
Env	envelope protein
FACS	fluorescence-activated cell sorter
FITC	fluoresceinisothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GR	glucocorticoid receptor
GTFs	general transcription factors
HAT	histone acetyltransferase
HDAC	histone deacetylase
HRE	hormone responsive element
HS1	Dnase I-hypersensitive site 1
ID	internal deletion
IgH	immunoglobulin heavy chain
IL-2Rα,	interleukin-2 receptor alpha
IN	integrase
IRES	internal ribosomal entry site
LTR	long terminal repeat

MAR	nuclear matrix-associated region
MARs	matrix attachment reagions
MBR	major breakpoint region
MMTV	mouse mammary tumor virus
MOI	multiplicity of infection
MTVR	MMTV receptor
NBP	NRE-binding protein
NF1	nuclear factor 1
NLS	nuclear localization signal
NM	nuclear matrix fraction
NMTS	nuclear matrix targeting sequence
NRE	negative regulatory element
Nuc	nucleosome
NuMA	nuclear-mitotic apparatus protein
NURD	nucleosome remodeling and deacetylase complex
Oct 1	octamer-binding protein 1
P/CAF	p300/CBP-associated factor
PBS	phosphate-buffered saline
PBS	primer-binding site
PMSF	phenylmethylsulfonyl fluoride
RGS12TS-S	regulator of G protein signaling
RNP	ribonucleoprotein
RNP pol II	RNA ploymerase II
RPA	ribonuclease protection assay
RPMI	Roswell park memorial institute
RT	reverse transcription
SA	splice acceptor
Sag	superantigen
SATB1	special AT-rich binding protein 1
SD	splice donor
SF	soluble fraction
siRNA	small interfering RNA.
SnRNP	small nuclear ribonucleoproteins
SRC	steroid receptor coactivator
STAT	signal transducers and activators of transcription
SUMO	small ubiquitin-related modifier
TBP	TATA-binding protein
TCR	T-cell receptor
Tfrc	transferrin receptor
TM	transmembrane
TNFα	tumor necrosis factor α
TRAP	thyroid hormone receptor-associated protein
TSA	trichostatin A
UBP	upper or ubiquitous-binding protein
WL	whole cell lysate

YY1 ying-yang 1

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